BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 3334–3338, April 1993
Chemistry

Spontaneous assembly of a self-complementary oligopeptide to form a stable macroscopic membrane

(β-sheet/insoluble filaments/ionic bonds/origin of life/zuotin)

SHUGUANG ZHANG*†, TODD HOLMES‡, CURTIS LOCKSHIN*, AND ALEXANDER RICH*†

Departments of *Biology 16-739 and ‡Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139

Contributed by Alexander Rich, December 30, 1992

ABSTRACT **A 16-residue peptide [(Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys)$_2$] has a characteristic β-sheet circular dichroism spectrum in water. Upon the addition of salt, the peptide spontaneously assembles to form a macroscopic membrane. The membrane does not dissolve in heat or in acidic or alkaline solutions, nor does it dissolve upon addition of guanidine hydrochloride, SDS/urea, or a variety of proteolytic enzymes. Scanning EM reveals a network of interwoven filaments ≈10–20 nm in diameter. An important component of the stability is probably due to formation of complementary ionic bonds between glutamic and lysine side chains. This phenomenon may be a model for studying the insoluble peptides found in certain neurological disorders. It may also have implications for biomaterials and origin-of-life research.**

Peptides of alternating hydrophilic and hydrophobic amino acid residues have a tendency to adopt a β-sheet structure. The complete sequence of (Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys)$_2$ (EAK16) was originally found in a region of alternating hydrophobic and hydrophilic residues in zuotin, a yeast protein that was initially identified for its ability to bind preferentially to left-handed Z-DNA (1). Previous studies with alternating amphiphilic-peptide polymers—e.g., poly(Val-Lys), poly(Glu-Ala), poly(Tyr-Glu), poly(Lys-Phe), poly(Lys-Leu)—and oligopeptides [(Val-Glu-Val-Orn)$_{1-3}$]-Val (2–7) have shown that these polymers can adopt β-sheet structures and can aggregate, depending upon pH, salt, and time. However, self-complementary EAK16 is distinctive in that it forms an insoluble macroscopic membrane.

MATERIALS AND METHODS

Peptides. The Glu-Ala-Lys peptides were synthesized by a peptide synthesizer (Applied Biosystems), purified by reverse-phase HPLC, and eluted by a linear gradient of 5–80% acetonitrile/0.1% trifluoacetic acid. The peptide stock solutions were dissolved in water (1–5 mg/ml) or in 23% acetonitrile (10 mg/ml). The concentrations of the peptides were determined by dissolving dried peptide in water (wt/vol) and centrifuging the solution. A portion of the solution was then analyzed by hydrolysis with internal controls. The sequence of the peptides was confirmed by microsequencing. The composition of the peptides was confirmed by hydrolytic analysis. Ala-Glu-Ala-Lys-Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys (EAK12) and EAK16 are acetylated and aminated at the N- and C-terminal ends, respectively. Blocking of both N and C termini of EAK16 appears nonessential for membrane formation.

CD Measurement. CD spectra were gathered on an Aviv model 60DS spectropolarimeter with 60HDS software for data processing. Because EAK16 contains both positively and negatively charged residues, the peptide itself can serve as a buffer. CD samples were prepared by diluting stock peptide solution (1–5 mg/ml) in water.

Membrane Preparations. The membranes were prepared as follows: 5–10 μl of the stock solution of EAK16 peptide (1–5 mg/ml) was added to 0.5–1.0 ml of phosphate-buffered saline (150 mM NaCl/10 mM sodium phosphate, pH 7.4) with 0.00001% Congo red in a 24-well-microtiter plate. The membrane was photographed under an inverted optical microscope with a rule underneath it as a size reference. The samples for scanning EM were prepared by first incubating the membranes in 5% glutaraldehyde at 4°C for 30 min and then dehydrating them sequentially with 20, 50, 70, 90, and 100% ethanol and liquid CO_2. The specimen was examined by using scanning EM at ×400–×20,000 magnification.

RESULTS AND DISCUSSION

Properties of EAK16. CD studies of EAK16 indicate a typical spectrum of β-sheet formation with a minimum ellipticity at 218 nm and a maximum ellipticity at 195 nm (Fig. 1). Because of this form, the molecule has hydrophobic alanine side chains on one side and self-complementary pairs of positively charged lysine- and negatively charged glutamic acid-side chains on the other surface. EAK16 spontaneously associates to form a macroscopic membrane, whereas the 12-amino acid peptide Ala-Glu-Ala-Lys-Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys (EAK12) of similar composition can associate to a much smaller extent. This result suggests that alternating pairs of complementary ionic bonds may be important or that the structure has parallel β-sheets. Five other peptides with various compositions and lengths, including a single unit of the repeat Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys (EAK8), did not form a membrane under the same conditions (Table 1).

Macroscopic Membrane Formation. The spontaneous assembly of EAK16 was first observed serendipitously in Dulbecco modified Eagle's medium/calf serum when it was being tested for toxicity. EAK16 did not affect the growth rate of nerve growth factor-differentiated rat PC-12 cells and was apparently nontoxic (data not shown). However, a transparent membrane was seen when viewed under ×100 magnification phase-contrast microscopy; the membrane was also visible in phosphate-buffered saline (Fig. 2*A*). The membrane can be stained by Congo red (Fig. 2 *B* and *C*), a dye that preferentially stains β-pleated sheet structures and is commonly used to visualize abnormal protein deposition in tissues (10). However, other peptides listed in Table 1 did not form visible macroscopic membranes when tested.

Stability of the Membrane. Once the membrane is formed, it is stable and resistant to digestion with several proteases—including trypsin, α-chymotrypsin, papain, protease K, and pronase—at a concentration of 100 μg/ml, even though EAK16 contains potential protease-cleavage sites (Table 1).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: EAK16, (Ala-Glu-Ala-Glu-Ala-Lys-Ala-Lys)$_2$.

†To whom reprint requests should be addressed.

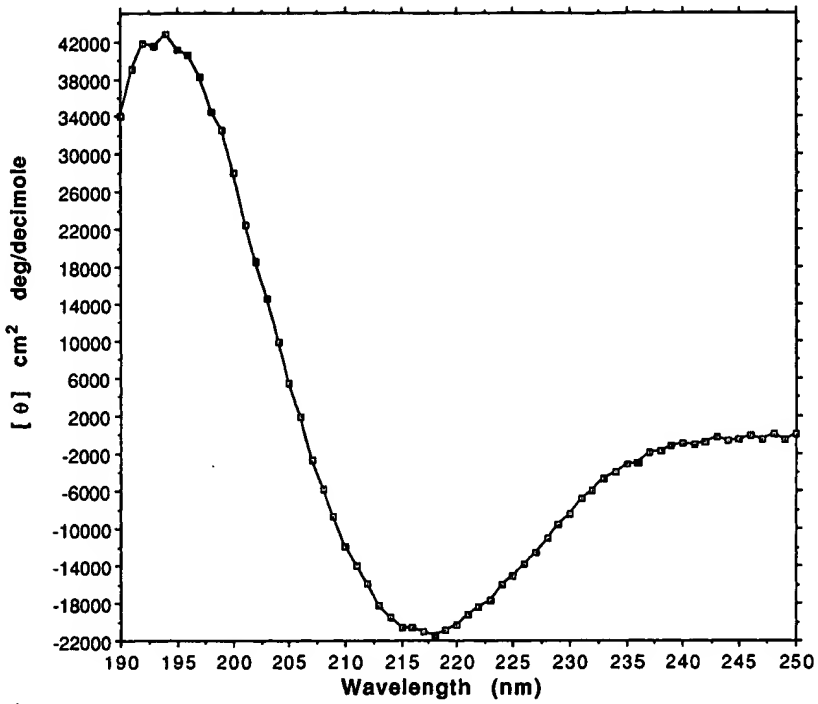

FIG. 1. CD spectrum of the EAK16 peptide. The EAK16 peptide was dissolved in water (10 μM) before taking the CD spectrum. A typical β-sheet CD spectrum with a 218-nm minimum and a 195-nm maximum is detected.

The membrane is stable in 1% SDS at 90°C for >4 hr. These observations are consistent with other studies that showed that the β-sheet CD spectrum was not significantly changed by heating the EAK16 solution to 90°C, by various pH (1.5, 3.0, 7.0, and 11), or by 0.1% SDS, 7 M guanidine hydrochloride, or 8 M urea (S.Z. and C.L., unpublished work). The membrane is mechanically stable and can be transferred from one solution to another by using a solid support but can be broken by cutting, tearing, or shearing.

Effect of Salts. Salt appears to play an important role in this spontaneous-assembly process. A variety of cations were tested. The order of effectiveness in inducing membrane formation appears to be $Li^+ > Na^+ > K^+ > Cs^+$. Cs^+ largely produces precipitates rather than a structural membrane. In aqueous solution, Li^+ has the largest hydrated radius (3.4 Å), whereas Na^+ (2.76 Å), K^+ (2.32 Å), and Cs^+ (2.28 Å) have smaller hydrated radii (11). The formation of the membrane seems to correlate with the order of the enthalpies of the monovalent metal ions (11). On the other hand, NH_4^+ and Tris·HCl seem not to induce EAK16 to form a membrane. Under our conditions, divalent metal ions primarily induced aggregates rather than membrane formation. At present, it is not known if the metal ions act as the catalyst or are themselves incorporated into the membrane, although the latter seems more likely.

There are numerous examples of monovalent metal ions that promote and stabilize other structures. One of these is the association of four guanosine nucleotides in nucleic acids called a G-quartet (12, 13). In this case, the order of the effectiveness is $K^+ > Na^+ > Cs^+ >>> Li^+$ for G-quartet formation (12). Brack and Orgel (2) reported that poly(Val-Lys) in water at pH 2.3 could be changed from a random coil to a stable β-sheet in high-molecular-weight aggregates in the presence of 100 mM NaCl. Furthermore, poly(Phe-Lys) and poly(Tyr-Lys), but not nonalternating peptides of similar compositions, can associate to form high-molecular-weight complexes in the presence of salt (4, 6). An additional example of salt-induced peptide aggregation is the β-amyloid

Table 1. Peptides used in this study

Peptide	Sequence*	DMEM†	PBS†	Water†	Structure‡	Ref.
EAK16	Ac-HN-AEAEAKAKAEAEAKAK-$CONH_2$	++++	++++	+	β	This study
EAK12	Ac-HN-AEAKAEAEAKAK-$CONH_2$	++	+	−	α, β	This study
EAK8	H_2N-AEAEAKAK-COOH	−	−	−	RC	This study
β-Amyloid-(1–28)	H_2N-DAEFRHDSGYEVHHQKLVFFAEDVGSNK-COOH	−	−	−	RC, α, β	8
β-Amyloid-(25–35)	H_2N-GSNKGAIIGLM-$CONH_2$	−	−	−	RC	This study
Substance P	H_2N-RPKPQQFGLM-$CONH_2$	−	−	−	ND	This study
Spantide	H_2N-(D)RPKPQQ(D)WL(D)L-$CONH_2$	−	−	−	ND	This study

EAK12 and EAK16 are acetylated and aminated at N- and C-terminal ends, respectively. Blocking of both N and C termini of EAK16 appears nonessential for membrane formation. A volume of 5–10 μl of dissolved peptide was applied to the Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), or water. Formation of the membrane-like structure was first observed under a phase-contrast microscope and then by naked eye. Sustance P, Spantide, and β-amyloid-(1–28) are commercially available from Bachem. The (D) in Spantide indicates D-amino acids incorporated into the peptide. Substance P, Spantide, and β-amyloid-(25–35) are aminated on the C-terminal ends.

*One-letter amino acid code is used.

†The + and − denote the presence or absence of the membranous structure, respectively.

‡α, α-Helix; β, β-sheet; RC, random coil; ND, structures not determined.

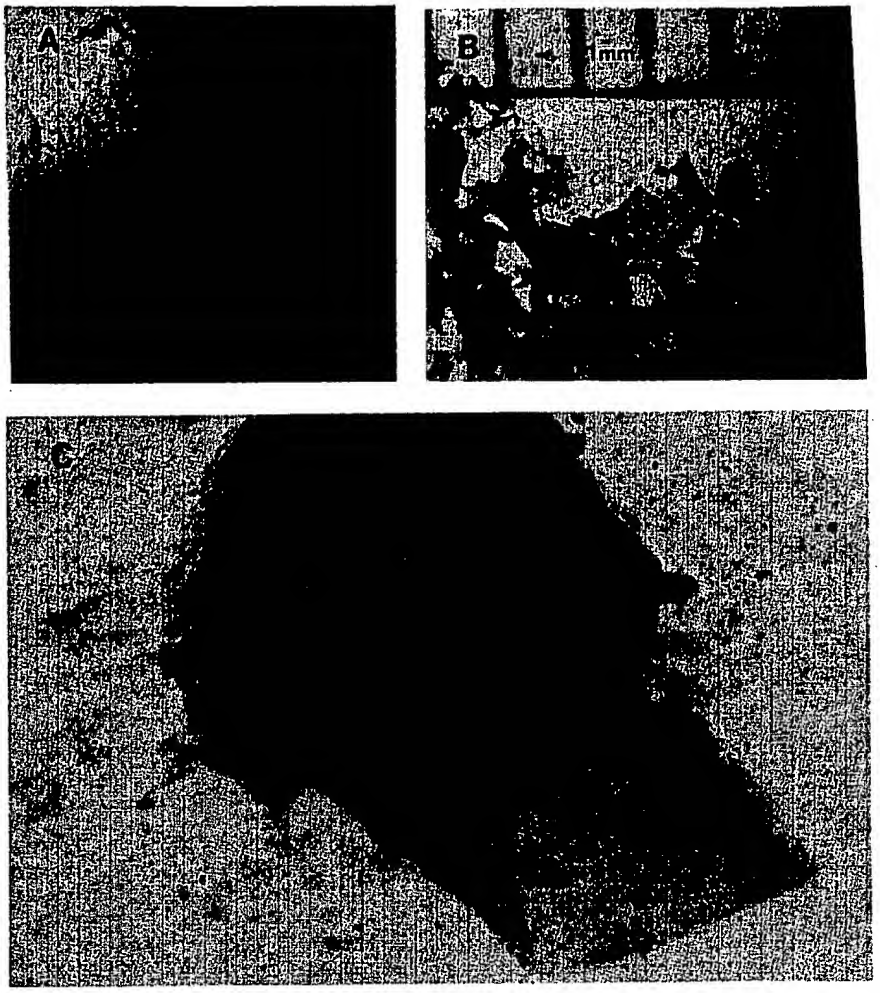

FIG. 2. Photographs of the membrane. (*A*) The structure was formed in phosphate-buffered saline and transferred to a glass slide. The colorless membranous structures are isobuoyant; therefore, the image is not completely in focus. (×75; Nomarski microscope.) (*B*) The structure stained bright red with Congo red (9) and can then be seen by the naked eye. (×15; each scale unit = 1 mm). (*C*) A portion of a well-defined membranous structure with layers is clearly visible; the dimensions of this particular membrane are ≈2 × 3 mm. (×20.)

protein found in the plaques of Alzheimer disease. The β-amyloid protein has 43-amino acid residues and is highly soluble in water (up to 30 mg/ml), but it is poorly soluble (0.5 mg/ml) in phosphate-buffered saline (14).

Scanning EM. The architecture of the membrane appears to resemble high-density felt. At low magnification (×20–×100), the structure looks like a flat membrane. However, structural details are revealed by scanning EM at high

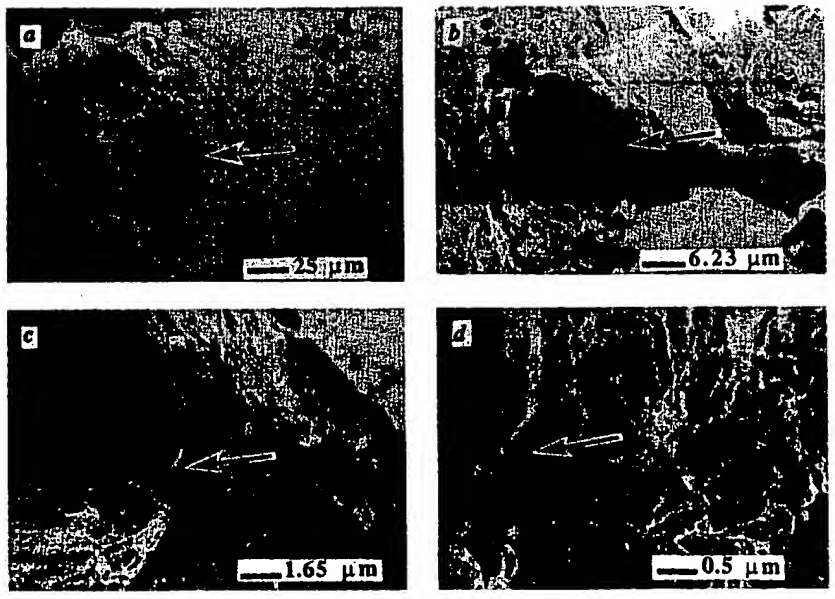

FIG. 3. Serial photography of scanning EM. The diameter of the filaments are ≈10–20 nm, and the distance between fibers are ≈50–80 nm. Arrows mark the same location. (*a*, ×300; *b*, ×1200; *c*, ×4500; *d*, ×15,000.)

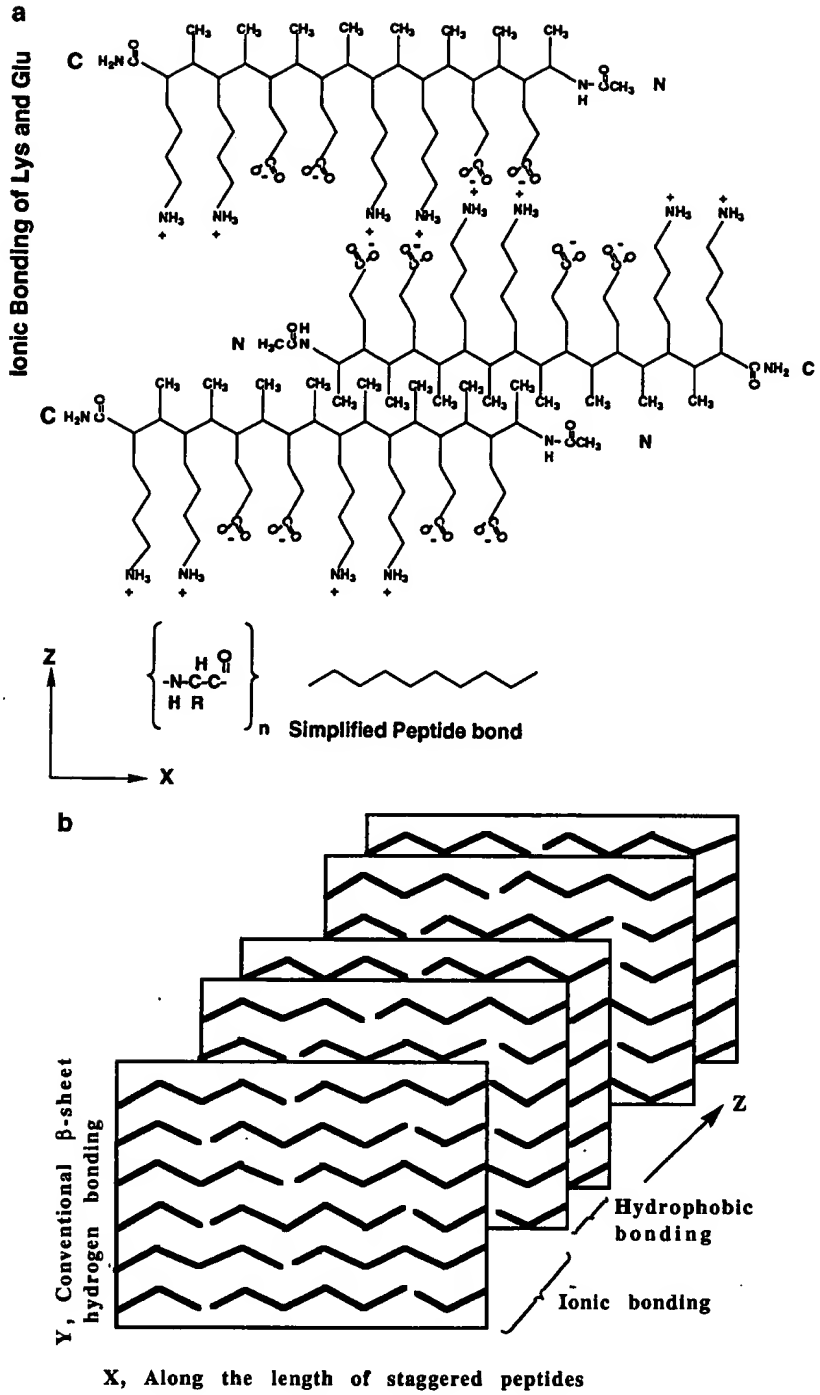

FIG. 4. Proposed model of the membrane. (*a*) View perpendicular to the β-sheet, which is the *y* axis. Three molecules of EAK16 peptide form three layers of an antiparallel β-sheet, held together on one side by hydrophobic bonding between alanine side chains facing each other and the charged lysines and glutamic acid side chains facing each other to form ionic bonds. The structure can also be drawn as a parallel β-sheet. In either case, the peptide would be staggered along *x*, as shown in the diagram. (*b*) Stacking of β-sheets. The staggered peptides are oriented along the *x* axis. The *z* axis has the complementary ionic bonds between lysine and glutamic acid, as well as the hydrophobic bonds between alanines referred to in *a*. The *y* axis contains the conventional β-sheet hydrogen bonds. Similar interactions are found in sawfly silk fibroin containing 70–80% alanine and glutamine but without the ionic pairing (21).

magnification (Fig. 3). The membrane appears formed from interwoven individual filaments. The self-complementary individual EAK16 oligopeptides probably interact strongly to form a stable structure promoted by the hydrated salt ions.

Other examples of oligopeptides forming insoluble filaments are found in several pathological diseases—e.g., the neurofibrillary tangles found in Alzheimer disease plaques form salt-dependent aggregates from β-amyloid protein with an

extremely stable β-sheet structure that stains with Congo red (15). At high magnification, the aggregated Alzheimer filaments have a diameter of ≈10–15 nm (14–17), similar to the EAK16 filaments. The scrapie prion protein likewise stains with Congo red and forms aggregated filaments that are extremely stable and resistant to proteases (18, 19). Peptides that spontaneously assemble to form an insoluble filament have also been reported in liver cirrhosis, where intracellular inclusions are found due to a mutation that occurs at the A β-sheet region of the Z α_1-antitrypsin (20).

Proposed Structure of the Membrane. EAK16 contains two-unit repeats of alternating hydrophilic and hydrophobic residues, where every other residue in the peptide is alanine. At neutral pH, the four glutamic acids and four lysines are negatively and positively charged, respectively. Because of the β-sheet structure, all of the charged amino acids lie on the same side and, thus, have complementary patterns with pairs of positively and negatively charged ionic groups. The peptides may be staggered, which would contribute to stability in the direction along the molecule (Fig. 4 *a* and *b*, *x* axis). The alanines on one side of the β-sheet form hydrophobic bonds, as in silk fibroin (21), and glutamic acids and lysines on the other side form complementary ionic bonds (Fig. 4 *a* and *b*, *z* axis). However, the peptides could also be organized in register, without staggering. The results of x-ray diffraction studies will be published elsewhere.

Implications of the Self-Complementary Peptide Structure. Spontaneous formation of such a macroscopic membrane has some implications for biology and for biomaterial research. Because of the extreme stability of the EAK16 membrane in serum, where it was originally discovered, its high resistance to proteolytic digestion, simple composition, apparent lack of cytotoxicity, and easy synthesis in large quantities, such materials might be useful for biomaterial applications. These could include slow-diffusion drug-delivery systems, artificial skin, and separation matrices.

The similarity of the EAK16 filaments to the insoluble proteins found in various pathological diseases (14–20) suggests that it might be a useful model system for exploring those aspects of structures and sequences that produce such unusual properties as extreme insolubility, resistance to proteolytic digestion, and spontaneous assembly. Drugs that inhibit self-assembly of the peptides may be useful for the treatment of these diseases.

It is of great interest that oligopeptides containing simple self-complementary repeats can spontaneously assemble to form relatively ordered macroscopic structures, independent of an external assembly mechanism or instruction code. Brack and Orgel (2) suggested that alternating peptides with a tendency to form β-sheets may be able to form "membrane-like aggregates." Our observation of a macroscopic membrane spontaneously assembled from EAK16 is consistent with the hypothesis that such simple molecules may form larger and more complex structures, which may have been important in the origin of life. Miller and Urey (22) have shown that amino acids are readily synthesized from CH_4, NH_3, H_2, and H_2O molecules and that amino acids can be condensed to form oligopeptides in prebiotic conditions (for review, see ref. 23). Further, Yanagawa *et al.* (9) reported that a 12-residue peptide of glycines can also form 30- to 50-μm microscopic aggregates of different shapes and textures, dependent on salt and other conditions and similar to our observations. We speculate that oligopeptides with self-complementary sequences, as in the example of EAK16, might serve as templates to condense tetra- or octapeptides of similar sequence to form longer oligopeptides. These oligopeptides could then spontaneously assemble to form membranes, yielding compartmentalization and eventually establish an enclosed environment for a primitive metabolism (24).

We thank Richard Cook and Michael Kelley for synthesis of peptides; Barbara Slack for suggesting use of Congo red; Beth Sawin, Gabriella Krochmalnic, and Patricia Reiley for helping with some of the photographs; and Dr. Chulhee Kang for helping with computer graphics. We also thank Drs. Martin Egli, Stafen Wölfl, Monty Krieger, Vincent Rotello, and Julius Rebek, Jr., for helpful discussions. This work is supported by grants from the National Institutes of Health, the National Science Foundation, the National Aeronautics and Space Administration, the Office of Naval Research, and the American Cancer Society. S.Z. was an American Cancer Society Postdoctoral Fellow.

1. Zhang, S., Lockshin, C., Herbert, A., Winter, E. & Rich, A. (1992) *EMBO J.* **11**, 3787–3796.
2. Brack, A. & Orgel, L. E. (1975) *Nature (London)* **256**, 383–387.
3. Rippon, W. B., Chen, H. H. & Walton, A. G. (1973) *J. Mol. Biol.* **75**, 369–375.
4. Seipke, G., Arfmann, H. A. & Wagner, K. G. (1974) *Biopolymers* **13**, 1621–1633.
5. Piggion, E., Cosani, A., Terbojevich, M. & Borin, G. (1972) *Biopolymers* **11**, 633–643.
6. St. Pierre, S., Ingwall, R. T., Varlander, M. S. & Goodman, M. (1978) *Biopolymers* **17**, 1837–1847.
7. Osterman, D. G. & Kaiser, E. T. (1985) *J. Cell. Biochem.* **29**, 57–72.
8. Barrow, C. J. & Zagorski, M. G. (1991) *Science* **253**, 179–182.
9. Yanagawa, H., Nishizawa, M. & Kojima, K. (1984) *Origins Life* **14**, 267–272.
10. Pears, A. G. E. (1960) *Histochemistry: Theoretical and Applied* (Little, Brown, Boston), 2nd Ed.
11. Pauling, L. (1960) *Nature of the Chemical Bond and the Structure of Molecules and Crystals: An Introduction to Model Structural Chemistry* (Cornell Univ. Press, Ithaca, NY), 3rd Ed.
12. Williamson, J. R., Raghuraman, M. K. & Cech, T. R. (1989) *Cell* **59**, 871–880.
13. Kang, C.-H., Zhang, X., Ratliff, R., Moyzis, R. & Rich, A. (1992) *Nature (London)* **356**, 126–131.
14. Hilbich, C., Kisters-Woike, B., Reed, J., Masters, C. L. & Beyreuther, K. (1991) *J. Mol. Biol.* **50**, 149–165.
15. Iqbal, K. & Wisniewski, H. M. (1983) in *Alzheimer's Disease: The Standard Reference*, ed. Reisberg, B. (Free Press, Collier Macmillan, London), pp. 48–56.
16. Halverson, K., Fraser, P. E., Kirschner, D. A. & Lansbury, P. T., Jr. (1990) *Biochemistry* **29**, 2639–2644.
17. Kirschner, D. A., Inouye, H., Duffy, L. K., Sinclair, A., Lind, M. & Selkoe, D. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6953–6957.
18. Prusiner, S. B., Mckinley, M. P., Bowman, K. A., Bolton, D. C., Bendhein, P. E., Groth, D. F. & Glenner, G. G. (1983) *Cell* **35**, 349–358.
19. Brown, P., Liberski, P. P., Wolff, A. & Gajdusek, C. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7240–7244.
20. Lomas, D. A., Evans, D. L., Finch, J. T. & Carrell, R. W. (1992) *Nature (London)* **357**, 605–607.
21. Lucas, F. & Rudall, K. M. (1968) in *Comprehensive Biochemistry*, eds. Florikin, M. & Stotz, F. H. (Elsevier, Amsterdam), Vol. 26B, pp. 475–558.
22. Miller, S. L. & Urey, H. C. (1959) *Science* **130**, 245–251.
23. Chyba, C. & Sagan, C. (1992) *Nature (London)* **355**, 125–132.
24. Bernal, J. D. (1965) in *The Origins of Prebiological Systems and of Their Molecular Matrices*, ed. Fox, S. W. (Academic, New York), pp. 65–88.

EXHIBIT B

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61L 27/00, C12N 5/00 — A1

(11) International Publication Number: WO 96/40304

(43) International Publication Date: 19 December 1996 (19.12.96)

(21) International Application Number: PCT/US96/09065

(22) International Filing Date: 5 June 1996 (05.06.96)

(30) Priority Data:
478,690 7 June 1995 (07.06.95) US

(71) Applicant: REPROGENESIS, INC. [US/US]; 2401 Distribution Street, Charlotte, NC 28203 (US).

(72) Inventor: HUBBELL, Jeffrey, A.; 1020 Darby Road, San Marino, CA 91108 (US).

(74) Agent: PABST, Patrea, L.; Arnall Golden & Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GE 30309-3450 (US).

(81) Designated States: AL, AM, AU, BB, BG, BR, CA, CN, CZ, EE, FI, GE, HU, IL, IS, JP, KG, KP, KR, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: INJECTABLE HYDROGEL COMPOSITIONS

(57) Abstract

Slowly polymerizing hydrogels are provided which are useful as a means of delivering large numbers of isolated cells via injection. The gels promote engraftment and provide three-dimensional templates for new cell growth. The resulting tissue is similar in composition and histology to naturally occurring tissue. This method can be used for a variety of reconstructive procedures, including custom molding of cell implants to reconstruct three-dimensional tissue defects, as well as implantation of tissues generally. The polymers permit construction of muscle and cartilage tissues which are useful to repair defects such as reflux and incontinence.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

INJECTABLE HYDROGEL COMPOSITIONS

Background of the Invention

The present invention is generally in the area of using polymeric hydrogel-cell compositions in medical treatments.

Craniofacial contour deformities

Craniofacial contour deformities, whether traumatic, congenital, or aesthetic, currently require invasive surgical techniques for correction. Furthermore, deformities requiring augmentation often necessitate the use of alloplastic prostheses which suffer from problems of infection and extrusion. A minimally invasive method of delivering additional autogenous cartilage or bone to the craniofacial skeleton would minimize surgical trauma and eliminate the need for alloplastic prostheses. If one could transplant via injection and cause to engraft large numbers of isolated cells, one could augment the craniofacial osteo-cartilaginous skeleton with autogenous tissue, but without extensive surgery.

Unfortunately, attempts to inject dissociated cells subcutaneously or to implant dissociated tissues within areas of the body such as the peritoneum have not been successful. Cells are relatively quickly removed, presumably by phagocytosis and cell death.

Cells can be implanted onto a polymeric matrix and implanted to form a cartilaginous structure, as described in U.S. Patent No. 5,041,138 to Vacanti, et al., but this requires surgical implantation of the matrix and shaping of the matrix prior to implantation to form a desired anatomical structure.

Vesicoureteral reflux.

Vesicoureteral reflux is a condition wherein there is an abnormal development of the ureteral bud as it enters the bladder during embryologic development. The shortened course of the ureter through the bladder musculature decreases the ureteral resistance and allows for urine to reflux from the bladder reservoir back up into the ureter and into the kidney. With this condition, bacteria which may occasionally be present in the bladder through retrograde urethral transport, can reach the kidneys

and cause recurrent pyelonephritis. In addition, the constant back pressure of the urine into the calyces and renal pyramids results in mechanical damage to the renal parenchyma. If untreated, urinary vesicoureteral reflux can cause loss of renal parenchyma, and in some instances, renal failure, as reviewed by Atala and Casale, Infections in Urology 39-43 (March/April 1990). In 1960, 70% of the patients with renal failure were described as having vesicoureteral reflux as the primary etiology. With the advent of new diagnostic and treatment modalities, patients with vesicoureteral reflux now account for less than 1% of the renal failure population.

In the past, vesicoureteral reflux was usually diagnosed with a voiding cystogram after the child presented with repeated episodes of pyelonephritis. With the increased use of prenatal and postnatal sonography, hydronephrosis is more detectable, prompting further radiologic workup and earlier detection, as reported by Atala and Casale. Vesicoureteral reflux is graded depending on the severity. Grade 1 reflux signifies that urine is seen refluxing from the bladder up to the ureter only; in grade 2 reflux, urine refluxes into the ureter and calyceal dilatation. Grade 4 and 5 reflux are more severe, showing ureteral tortuosity and further calyceal blunting and dilatation, respectively.

The treatment of vesicioureteral reflux has been well established over the last decade. Initially it was believed that all patients with reflux would require surgery. Another school of management soon proposed that only medical therapy with antibiotics was required. It is now well established that the treatment of reflux depends on many factors, including the severity of reflux, associated congenital abnormalities, and the social situation of the child (parental compliance with medical treatment). Medical treatment is usually recommended for patients with grade 1 and 2 reflux, which usually resolve on their own as the bladder/ureteral configuration changes with growth. Grade 3 reflux is generally managed with medical therapy unless it persists or breakthrough

infections occur while on antibiotic suppression. Surgical treatment is usually required for grade 4 and 5 reflux.

Medical treatment implies that the patient is treated with daily suppressive antibiotics. A close follow-up is required in these patients, generally consisting of a catheterized urine culture every three months, an ultrasound exam and serum analysis every six months, a fluoroscopic or nuclear voiding cystourethrogram every year, and a DMSA renal scan every two years. Surgical treatment consists of an open surgery wherein a low abdominal incision is made, the bladder is entered, the ureters are mobilized and new ureteral submucosal tunnels are created; thereby extending the muscular backing of the ureter which increases their resistance. These patients require a general endotracheal anesthetic for a four to five hour surgery, an epidural catheter for both intraoperative and postoperative pain control, a bladder catheter for drainage, a perivesical drain, and a five to six day hospital stay. Antibiotic therapy and bladder antispasmodics are required post-operatively.

Although open surgical procedures for the correction of reflux have excellent results in the hands of experienced surgeons, it is associated with a well recognized morbidity, including pain and immobilization of a lower abdominal incision, bladder spasms, hematuria, and post-operative voiding frequency in some children. In an effort to avoid open surgical intervention, widespread interest was initiated by Matouschek's clinical experience with the endoscopic injection of Teflon™ (polytetrafluoroethylene) paste subureterally in 1984, as reported in Matouschek, E.: Die Behandlung des vesikorenalen Refluxes durch transueterale Einspritzung von polytetrafluoroethylenepast. Urologe, 20:263 (1981). With this technique, a cystoscope is inserted into the bladders, a needle is inserted through the cystoscope and placed under direct vision underneath the refluxing ureter in the submucosal space, and Teflon™ paste is injected until the gaping ureteric orifice configuration changes into a half-moon slit. The Teflon™ paste, injected endoscopically, corrects the reflux by acting as a bulking material which

increases ureteral resistance. However, soon after the introduction of this treatment, a controversy regarding the use of Teflon™ paste ensued. Malizia et al. "Migration and granulomatous reaction after periurethral injection of polymer (polytetrafluoroethylene)" JAMA, 251:3277 (1984), showed granuloma formation and particulate migration to the brain, lungs, and lymph nodes in animal studies. Polytetrafluoroethylene migration and granuloma formation have also been reported in humans by Claes et al., "Pulmonary migration following periurethral polyetrafluoroethylene injection for urinary incontinence" J. Urol., 142:821 (1989). The safety of Teflon™ for human use was questioned, and the paste was thereafter banned by the FDA.

However, there are definite advantages in treating vesicoureteral reflux endoscopically. The method is simple and can be completed in less than fifteen minutes, it has a success rate of greater than 85% with low morbidity and it can be performed in an outpatient basis, as reported by Atala et al, "Endoscopic treatment of vesicoureteral reflux with a self-detachable balloon system" J. Urol. 148:724 (1992). The goal of several investigators has been to find alternate implant materials which would be safe for human use.

Bovine dermal collagen preparations have been used to treat reflux endoscopically. However, only 58.5% of the patients were cured at one year follow-up, as described by Leonard et al, "Endoscopic injection of glutaraldehyde cross-linked bovine dermal collagen for correction of vesicoureteral reflux" J. Urol. 145:115 (1991). The collagen implant volume decreases with time, which results in high percentage of recurrence of reflux, over 90% within 3 years. The high failure rate with this substance presents a high risk to the unaware patient of developing renal damage after treatment.

A paste consisting of textured microparticles of silicone, suspended in a hydrogel, has been injected subureterally to correct reflux with an initial success rate of 91% in one European study, as reported by Buckley at al., "Endoscopic correction of vesicoureteric reflux with injectable

silicone microparticles" J. Urol. 149: 259A (1993). However, distant particle migration has been observed in animal models, as reported by Henly et al., "Particulate silicone for use in periurethral injections: a study of local tissue effects and a search for migration" J. Urol. 147:376A (1992). Approximately thirty percent of the silicone particles have a diameter which is less than 100 μm. This suggests that thirty percent of the silicone particles have a potential for distant organ migration through the macrophage system. The manufacturer of this technology tried unsuccessfully to obtain FDA approval, and subsequently filed for bankruptcy.

Laparoscopic correction of reflux has been attempted in both an animal model (Atala et al, "Laparoscopic correction of vesicoureteral reflux" J. Urol. 150:748 (1993)) and humans (Atala, "Laparoscopic treatment of vesicoureteral reflux" Dial Ped Urol 14:212 (1993)) and is technically feasible. However, at least two surgeons with laparoscopic expertise are needed, the length of the procedure is much longer than with open surgery, the surgery is converted from an extraperitoneal to an intraperitoneal approach, and the cost is higher due to both increased operative time and the expense of the disposable laparoscopic equipment.

Despite the fact that over a decade has transpired since the Teflon™ controversy, little progress has been made in this area of research. The ideal substance for the endoscopic treatment of reflux should be injectable, non-antigenic, non-migratory, volume stable, and safe for human use (Atala et al, 1992).

Urinary incontinence.

Urinary Incontinence is the most common and the most intractable of all GU maladies. Urinary incontinence, or the inability to retain urine and not void urine involuntarily, is dependent on the interaction of two sets of muscles. One is the detrusor muscle, a complex of longitudinal fibers forming the external muscular coating of the bladder. The detrusor is activated by parasympathetic nerves. The second muscle is the smooth/striated muscle of the bladder sphincter. The act of voiding

requires the sphincter muscle be voluntarily relaxed at the same time that the detrusor muscle of the bladder contracts. As a person ages, his ability to voluntarily control the sphincter muscle is lost in the same way that general muscle tone deteriorates with age. This can also occur when a radical event such as paraplegia "disconnects" the parasympathetic nervous system causing a loss of sphincter control. In different patients, urinary incontinence exhibits different levels of severity and is classified accordingly.

The most common incontinence, particular in the elderly, is urge incontinence. This type of incontinence is characterized by an extremely brief warning following by immediate urination. This type of incontinence is caused by a hyperactive detrusor and is usually treated with "toilet training" or medication. Reflex incontinence, on the other hand, exhibits no warning and is usually the result of an impairment of the parasympathetic nerve system such as a spinal cord injury.

Stress incontinence is most common in elderly women but can be found in women of any age. It is also commonly seen in pregnant women. This type of incontinence accounts for over half of the total number of cases. It is also found in men but at a lower incidence. Stress incontinence is characterized by urine leaking under conditions of stress such as sneezing, laughing or physical effort. There are five recognized categories of severity of stress incontinence, designated as types as 0, 1, 2a, 2b, and 3. Type 3 is the most severe and requires a diagnosis of intrinsic Sphincter Deficiency or ISD (Contemporary Urology, March 1993). There are many popular treatments including weight loss, exercise, medication and in more extreme cases, surgical intervention. The two most common surgical procedures involve either elevating the bladder neck to counteract leakage or constructing a lining from the patient's own body tissue or a prosthetic material such as PTFE to put pressure on the urethra. Another option is to use prosthetic devices such as artificial sphincters to external devices such as intravaginal balloons or penile clamps. For treatment of type 3 stress incontinence, there has been

a recent trend toward injection of Teflon™ or collagen paste around the sphincter muscle in order to "beef up" the area and improve muscle tone. None of the above methods of treatment, however, are very effective for periods in excess of a year.

Overflow incontinence is caused by anatomical obstructions in the bladder or underactive detrustors. It is characterized by a distended bladder which leads to frequent urine leakage. This type of incontinence is treated acutely by catheterization and long-term by drug therapy. Enuresis or bed-wetting is a problem in pediatrics and is controlled by various alarming devices and pads with sensors. Enuresis is not considered a serious problem unless it lasts beyond the age of four or five. Finally, there is true functional incontinence which occurs in patients with chronic impairment either of mobility or mental function. Such patients are usually treated by the use of diapers, incontinence pads or continuous catheterization (BBI, 1985 Report 7062).

WO 94/25080 describes the use of injectable polysaccharide-cell compositions for delivering isolated cells by injection. There is a need for improved injectable polymer-cell compositions which are biocompatible and biodegradable for delivering isolated cells by injection or implantation.

Accordingly, it is an object of the present invention to provide methods and compositions for injection of cells to form cellular tissues and cartilaginous structures.

It is a further object of the invention to provide improved compositions to form cellular tissues and cartilaginous structures including non-cellular material which will degrade and be removed to leave tissue or cartilage that is histologically and chemically the same as naturally produced tissue or cartilage.

It is another object of the present invention to provide improved methods and materials for treating vesicoureteral reflux, incontinence, and other defects which results in a natural and permanent cure to the defect.

It is a further object of the present invention to provide methods and materials for treating vesicoureteral reflux, incontinence, and other defects which is quick, simple, safe, and relatively non-invasive.

Summary of the Invention

Slowly polymerizing, biocompatible, biodegradable hydrogels are provided which are useful for delivering large numbers of isolated cells into a patient to create an organ equivalent or tissue such as cartilage. The gels promote engraftment and provide three dimensional templates for new cell growth. The resulting tissue is similar in composition and histology to naturally occurring tissue. In one embodiment, cells are suspended in a polymer solution and injected directly into a site in a patient, where the polymer crosslinks to form a hydrogel matrix having cells dispersed therein. In a second embodiment, cells are suspended in a polymer solution which is poured or injected into a mold having a desired anatomical shape, then crosslinked to form a hydrogel matrix having cells dispersed therein which can be implanted into a patient. Ultimately, the hydrogel degrades, leaving only the resulting tissue.

This method can be used for a variety of reconstructive procedures, including custom molding of cell implants to reconstruct three dimensional tissue defects, as well as implantation of tissues generally.

In another embodiment, a method of treatment of vesicoureteral reflux, incontinence and other defects is provided wherein bladder muscle cells are mixed with a liquid polymeric material, to form a cell suspension, which is injected into the area of the defect, in an amount effective to yield a tissue that corrects the defect, for example, which provides the required control over the passage of urine. In one embodiment, human bladder muscle specimens or chondrocytes are obtained and processed, the cells are mixed with the polymer, which is designed to solidify at a controlled rate when contacted with a crosslinking agent, and then the cells are injected at the desired site where they proliferate and correct the defect.

Detailed Description of the Invention

Techniques of tissue engineering employing biocompatible polymer scaffolds hold promise as a means of creating alternatives to prosthetic materials currently used in craniomaxillofacial surgery, as well as formation of organ equivalents to replaced diseased, defective, or injured tissues. However, polymers used to create these scaffolds, such as polylactic acid, polyorthoesters, and polyanhydrides, are difficult to mold and hydrophobic, resulting in poor cell attachment. Moreover, all manipulations of the polymers must be performed prior to implantation of the polymeric material.

Biocompatible polymers described herein such as polysaccharides can form hydrogels which are malleable and can be used to encapsulate cells. To form a hydrogel containing the cells, a polymer solution is mixed with the cells to be implanted to form a suspension. Then, in one embodiment, the suspension is injected directly into a patient prior to crosslinking of the polymer to form the hydrogel containing the cells. The hydrogel forms over a short period of time. In a second embodiment, the suspension is injected or poured into a mold, where it crosslinks to form a hydrogel of the desired anatomical shape having cells dispersed therein which then may be implanted in a patient. The hydrogel may be produced, for example, by cross-linking a polysaccharide polymer by exposure to a monovalent cation. Other polymers capable of forming hydrogels may be used as disclosed herein, including modified alginate derivatives. In the embodiment where the polymer is crosslinked by contact with a crosslinking agent, the strength of the crosslink may be increased or reduced by adjusting the concentration of the polymer and/or crosslinking agent.

Source of Cells

Cells can be obtained directed from a donor, from cell culture of cells from a donor, or from established cell culture lines. In the preferred embodiment, cells of the same species and preferably immunological profile are obtained by biopsy, either from the patient or a close relative,

which are then grown to confluence in culture using standard conditions and used as needed. If cells that are likely to elicit an immune reaction are used, such as human muscle cells from immunologically distinct individual, then the recipient can be immunosuppressed as needed, for example, using a schedule of steroids and other immunosuppressant drugs such as cyclosporine. However, in the most preferred embodiment, the cells are autologous.

In the preferred embodiments, cells are obtained directly from a donor, washed and implanted directly in combination with the polymeric material. The cells are cultured using techniques known to those skilled in the art of tissue culture. Cells obtained by biopsy are harvested and cultured, passaging as necessary to remove contaminating cells. Isolation of chondrocytes and muscle cells is demonstrated in WO 94/25080, the disclosure of which is incorporated herein.

Cell attachment and viability can be assessed using scanning electron microscopy, histology, and quantitative assessment with radioisotopes. The function of the implanted cells can be determined using a combination of the above-techniques and functional assays. For example, in the case of hepatocytes, *in vivo* liver function studies can be performed by placing a cannula into the recipient's common bile duct. Bile can then be collected in increments. Bile pigments can be analyzed by high pressure liquid chromatography looking for underivatized tetrapyrroles or by thin layer chromatography after being converted to azodipyrroles by reaction with diazotized azodipyrroles ethylanthranilate either with or without treatment with P-glucuronidase. Diconjugated and monoconjugated bilirubin can also be determined by thin layer chromatography after alkalinemethanolysis of conjugated bile pigments. In general, as the number of functioning transplanted hepatocytes increases, the levels of conjugated bilirubin will increase. Simple liver function tests can also be done on blood samples, such as albumin production.

Analogous organ function studies can be conducted using techniques known to those skilled in the art, as required to determine the extent of cell function after implantation. For example, islet cells of the pancreas may be delivered in a similar fashion to that specifically used to implant hepatocytes, to achieve glucose regulation by appropriate secretion of insulin to cure diabetes. Other endocrine tissues can also be implanted. Studies using labelled glucose as well as studies using protein assays can be performed to quantitate cell mass on the polymer scaffolds. These studies of cell mass can then be correlated with cell functional studies to determine what the appropriate cell mass is. In the case of chondrocytes, function is defined as providing appropriate structural support for the surrounding attached tissues.

This technique can be used to provide multiple cell types, including genetically altered cells, within a three-dimensional scaffolding for the efficient transfer of large number of cells and the promotion of transplant engraftment for the purpose of creating a new tissue or tissue equivalent. It can also be used for immunoprotection of cell transplants while a new tissue or tissue equivalent is growing by excluding the host immune system.

Examples of cells which can be implanted as described herein include chondrocytes and other cells that form cartilage, osteoblasts and other cells that form bone, muscle cells, fibroblasts, and organ cells. As used herein, "organ cells" includes hepatocytes, islet cells, cells of intestinal origin, cells derived from the kidney, and other cells acting primarily to synthesize and secret, or to metabolize materials.

Addition of Biologically Active Materials to the hydrogel.

The polymeric matrix can be combined with humoral factors to promote cell transplantation and engraftment. For example, the polymeric matrix can be combined with angiogenic factors, antibiotics, antiinflammatories, growth factors, compounds which induce differentiation, and other factors which are known to those skilled in the art of cell culture.

For example, humoral factors could be mixed in a slow-release form with the cell-polymer suspension prior to formation of implant or transplantation. Alternatively, the hydrogel could be modified to bind humoral factors or signal recognition sequences prior to combination with isolated cell suspension.

Polymer Solutions

Polymeric materials which are capable of forming a hydrogel are utilized. The polymer is mixed with cells for implantation into the body and is permitted to crosslink to form a hydrogel matrix containing the cells either before or after implantation in the body. In one embodiment, the polymer forms a hydrogel within the body upon contact with a crosslinking agent. A hydrogel is defined as a substance formed when an organic polymer (natural or synthetic) is crosslinked via covalent, ionic, or hydrogen bonds to create a three-dimensional open-lattice structure which entraps water molecules to form a gel. Naturally occurring and synthetic hydrogel forming polymers, polymer mixtures and copolymers may be utilized as hydrogel precursors.

Examples of materials which can be used to form a hydrogel include modified alginates. Alginate is a carbohydrate polymer isolated from seaweed, which can be crosslinked to form a hydrogel by exposure to a divalent cation such as calcium, as described, for example in WO 94/25080, the disclosure of which is incorporated herein by reference. The modified alginate solution is mixed with the cells to be implanted to form a suspension. Then the suspension is injected directly into a patient prior to crosslinking of the polymer to form the hydrogel containing the cells. The suspension then forms a hydrogel over a short period of time due to the presence *in vivo* of physiological concentrations of calcium ions.

Alginate is ionically crosslinked in the presence of divalent cations, in water, at room temperature, to form a hydrogel matrix. Due to these mild conditions, alginate has been the most commonly used polymer for hybridoma cell encapsulation, as described, for example, in

U.S. Patent No. 4,352,883 to Lim. In the Lim process, an aqueous solution containing the biological materials to be encapsulated is suspended in a solution of a water soluble polymer, the suspension is formed into droplets which are configured into discrete microcapsules by contact with multivalent cations, then the surface of the microcapsules is crosslinked with polyamino acids to form a semipermeable membrane around the encapsulated materials.

Modified alginate derivatives may be synthesized which have an improved ability to form hydrogels. The use of alginate as the starting material is advantageous because it is available from more than one source, and is available in good purity and characterization. As used herein, the term "modified alginates" refers to chemically modified alginates with modified hydrogel properties. Naturally occurring alginate may be chemical modified to produce alginate polymer derivatives that degrade more quickly. For example, alginate may be chemically cleaved to produce smaller blocks of gellable oligosaccharide blocks and a linear copolymer may be formed with another preselected moiety, e.g. lactic acid or ϵ-caprolactone. The resulting polymer includes alginate blocks which permit ionically catalyzed gelling, and oligoester blocks which produce more rapid degradation depending on the synthetic design. Alternatively, alginate polymers may be used, wherein the ratio of mannuronic acid to guluronic acid does not produce a firm gel, which are derivatized with hydrophobic, water-labile chains, e.g., oligomers of ϵ-caprolactone. The hydrophobic interactions induce gelation, until they degrade in the body.

Additionally, polysaccharides which gel by exposure to monovalent cations, including bacterial polysaccharides, such as gellan gum, and plant polysaccharides, such as carrageenans, may be crosslinked to form a hydrogel using methods analogous to those available for the crosslinking of alginates described above. Polysaccharides which gel in the presence of monovalent cations form hydrogels upon exposure, for example, to a solution comprising physiological levels of sodium. Hydrogel precursor

solutions also may be osmotically adjusted with a nonion, such as mannitol, and then injected to form a gel.

Polysaccharides that are very viscous liquids or are thixotropic, and form a gel over time by the slow evolution of structure, are also useful. For example, hyaluronic acid, which forms an injectable gel with a consistency like a hair gel, may be utilized. Modified hyaluronic acid derivatives are particularly useful. As used herein, the term "modified hyaluronic acids" refers to chemically modified hyaluronic acids. Modified hyaluronic acids may be designed and synthesized with preselected chemical modifications to adjust the rate and degree of crosslinking and biodegradation. For example, modified hyaluronic acids may be designed and synthesized which are esterified with a relatively hydrophobic group such as propionic acid or benzylic acid to render the polymer more hydrophobic and gel-forming, or which are grafted with amines to promote electrostatic self-assembly. Modified hyaluronic acids thus may be synthesized which are injectable, in that they flow under stress, but maintain a gel-like structure when not under stress. Hyaluronic acid and hyaluronic derivatives are available from Genzyme, Cambridge, MA and Fidia, Italy.

Other polymeric hydrogel precursors include polyethylene oxide-polypropylene glycol block copolymers such as Pluronics™ or Tetronics™, which are crosslinked by hydrogen bonding and/or by a temperature change, as described in Steinleitner *et al.*, *Obstetrics & Gynecology*, 77:48-52 (1991); and Steinleitner *et al.*, *Fertility and Sterility*, 57:305-308 (1992). Other materials which may be utilized include proteins such as fibrin, collagen and gelatin. Polymer mixtures also may be utilized. For example, a mixture of polyethylene oxide and polyacrylic acid which gels by hydrogen bonding upon mixing may be utilized. In one embodiment, a mixture of a 5% w/w solution of polyacrylic acid with a 5% w/w polyethylene oxide (polyethylene glycol, polyoxyethylene) 100,000 can be combined to form a gel over the course of time, *e.g.*, as quickly as within a few seconds.

Covalently crosslinkable hydrogel precursors also are useful. For example, a water soluble polyamine, such as chitosan, can be cross-linked with a water soluble diisothiocyanate, such as polyethylene glycol diisothiocyanate. The isothiocyanates will react with the amines to form a chemically crosslinked gel. Aldehyde reactions with amines, e.g., with polyethylene glycol dialdehyde also may be utilized. A hydroxylated water soluble polymer also may be utilized.

Alternatively, polymers may be utilized which include substituents which are crosslinked by a radical reaction upon contact with a radical initiator. For example, polymers including ethylenically unsaturated groups which can be photochemically crosslinked may be utilized, as disclosed in WO 93/17669, the disclosure of which is incorporated herein by reference. In this embodiment, water soluble macromers that include at least one water soluble region, a biodegradable region, and at least two free radical-polymerizable regions, are provided. The macromers are polymerized by exposure of the polymerizable regions to free radicals generated, for example, by photosensitive chemicals and or light. Examples of these macromers are PEG-oligolactyl-acrylates, wherein the acrylate groups are polymerized using radical initiating systems, such as an eosin dye, or by brief exposure to ultraviolet or visible light. Additionally, water soluble polymers which include cinnamoyl groups which may be photochemically crosslinked may be utilized, as disclosed in Matsuda *et al.*, *ASAID Trans.*, 38:154-157 (1992).

In general, the polymers are at least partially soluble in aqueous solutions, such as water, buffered salt solutions, or aqueous alcohol solutions. Methods for the synthesis of the other polymers described above are known to those skilled in the art. See, for example Concise Encyclopedia of Polymer Science and Polymeric Amines and Ammonium Salts, E. Goethals, editor (Pergamen Press, Elmsford, NY 1980). Many polymers, such as poly(acrylic acid), are commercially available. Naturally occurring and synthetic polymers may be modified using chemical reactions available in the art and described, for example, in

March, "Advanced Organic Chemistry," 4th Edition, 1992, Wiley-Interscience Publication, New York.

Water soluble polymers with charged side groups may be crosslinked by reacting the polymer with an aqueous solution containing ions of the opposite charge, either cations if the polymer has acidic side groups or anions if the polymer has basic side groups. Examples of cations for cross-linking of the polymers with acidic side groups to form a hydrogel are monovalent cations such as sodium, and multivalent cations such as copper, calcium, aluminum, magnesium, strontium, barium, and tin, and di-, tri- or tetra-functional organic cations such as alkylammonium salts. Aqueous solutions of the salts of these cations are added to the polymers to form soft, highly swollen hydrogels and membranes. The higher the concentration of cation, or the higher the valence, the greater the degree of cross-linking of the polymer. Additionally, the polymers may be crosslinked enzymatically, *e.g.*, fibrin with thrombin.

In the embodiment wherein modified alginates and other anionic polymers that can form hydrogels which are malleable are used to encapsulate cells, the hydrogel is produced by cross-linking the polymer with the appropriate cation, and the strength of the hydrogel bonding increases with either increasing concentrations of cations or of polymer. Concentrations from as low as 0.001 M have been shown to cross-link alginate. Higher concentrations are limited by the toxicity of the salt.

The preferred anions for cross-linking of the polymers to form a hydrogel are monovalent, divalent or trivalent anions such as low molecular weight dicarboxylic acids, for example, terepthalic acid, sulfate ions and carbonate ions. Aqueous solutions of the salts of these anions are added to the polymers to form soft, highly swollen hydrogels and membranes, as described with respect to cations.

A variety of polycations can be used to complex and thereby stabilize the polymer hydrogel into a semi-permeable surface membrane. Examples of materials that can be used include polymers having basic

reactive groups such as amine or imine groups, having a preferred molecular weight between 3,000 and 100,000, such as polyethylenimine and polylysine. These are commercially available. One polycation is poly(L-lysine); examples of synthetic polyamines are: polyethyleneimine, poly(vinylamine), and poly(allyl amine). There are also natural polycations such as the polysaccharide, chitosan.

Polyanions that can be used to form a semi-permeable membrane by reaction with basic surface groups on the polymer hydrogel include polymers and copolymers of acrylic acid, methacrylic acid, and other derivatives of acrylic acid, polymers with pendant SO_3H groups such as sulfonated polystyrene, and polystyrene with carboxylic acid groups.

Cell Suspensions

Preferably the polymer is dissolved in an aqueous solution, preferably a 0.1 M potassium phosphate solution, at physiological pH, to a concentration forming a polymeric hydrogel, for example, for modified alginate, of between 0.5 to 2% by weight, e.g., 1%, modified alginate. The isolated cells are suspended in the polymer solution to a concentration of between 1 and 50 million cells/ml, most preferably between 10 and 20 million cells/ml.

Methods of Implantation

The techniques described herein can be used for delivery of many different cell types to achieve different tissue structures. In the preferred embodiment, the cells are mixed with the polymer solution and injected directly into a site where it is desired to implant the cells, prior to crosslinking of the polymer to form the hydrogel matrix. However, the matrix may also be molded and implanted in one or more different areas of the body to suit a particular application. This application is particularly relevant where a specific structural design is desired or where the area into which the cells are to be implanted lacks specific structure or support to facilitate growth and proliferation of the cells.

The site, or sites, where cells are to be implanted is determined based on individual need, as is the requisite number of cells. For cells

having organ function, for example, hepatocytes or islet cells, the mixture can be injected into the mesentery, subcutaneous tissue, retroperitoneum, properitoneal space, and intramuscular space. For formation of cartilage, the cells are injected into the site where cartilage formation is desired. One could also apply an external mold to shape the injected solution. Additionally, by controlling the rate of polymerization, it is possible to mold the cell-hydrogel injected implant like one would mold clay. Alternatively, the mixture can be injected into a mold, the hydrogel allowed to harden, then the material implanted.

The suspension can be injected via a syringe and needle directly into a specific area wherever a bulking agent is desired, i.e., a soft tissue deformity such as that seen with areas of muscle atrophy due to congenital or acquired diseases or secondary to trauma, burns, and the like. An example of this would be the injection of the suspension in the upper torso of a patient with muscular atrophy secondary to nerve damage.

The suspension can also be injected as a bulking agent for hard tissue defects, such as bone or cartilage defects, either congenital or acquired disease states, or secondary to trauma, burns, or the like. An example of this would be an injection into the area surrounding the skull where a bony deformity exists secondary to trauma. The injunction in these instances can be made directly into the needed area with the use of a needle and syringe under local or general anesthesia.

The suspension could also be injected percutaneously by direct palpation, such as by placing a needle inside the vas deferens and occluding the same with the injected bulking substance, thus rendering the patient infertile. The suspension could also be injected through a catheter or needle with fluoroscopic, sonographic, computed tomography, magnetic resonance imaging or other type of radiologic guidance. This would allow for placement or injection of this substance either by vascular access or percutaneous access to specific organs or other tissue regions in the body, wherever a bulking agent would be required.

Further, this substance could be injected through a laparoscope or thoracoscope to any intraperitoneal or extraperitoneal or thoracic organ. For example, the suspension could be injected in the region of the gastro-esophageal junction for the correcting of gastroesophageal reflux. This could be performed either with a thoracoscope injecting the substance in the esophageal portion of the gastroesophageal region, or via a laparoscope by injecting the substance in the gastric portion of the gastroesophageal region, or by a combined approach.

Vesicoureteral reflux is one of the most common congenital defects in children, affecting approximately 1% of the population. Although all patients do not require surgical treatment, it is still one of the most common procedure performed in children. Over 600 ureteral reimplants are performed yearly at Children's Hospital in Boston, Massachusetts. This translates to an approximately saving of 3600 inpatient hospital days per year at this institution alone, if the endoscopic treatment described herein is used instead of open surgery.

In addition to its use for the endoscopic treatment of reflux, the system of injectable autologous muscle cell may also be applicable for the treatment of other medical conditions, such as urinary and rectal incontinence, dysphonia, plastic reconstruction, and wherever an injectable permanent biocompatible material is needed. Methods for using an injectable polymer for delivering isolated cells via injection are described for example in WO 94/25080.

Improved injectable biocompatible polymers are disclosed herein which are useful for example as a delivery vehicle for muscle cells or chondrocytes in the treatment of reflux and incontinence. In one exemplary embodiment, a biopsy is obtained under anesthesia from a patient with vesicoureteral reflux, the isolated cells are mixed with a polymer capable of crosslinking to form a hydrogel, and the cell-polymer solution is injected endoscopically in the sub-ureteral region to correct reflux. The time to solidification of the polymer-cell solution may be manipulated by varying the concentration of the crosslinking agent as well

as the temperature at which the cells are added to the polymer. The use of autologous cells precludes an immunologic reaction. Solidification of the polymer impedes its migration until after it is degraded. The suspension can be injected through a cystoscopic needle, having direct visual access with a cystoscope to the area of interest, such as for the treatment of vesico-ureteral reflux or urinary incontinence.

In addition to the use of the cell-polymer suspension for the treatment of reflux and incontinence, the suspension can also be applied to reconstructive surgery, as well as its application anywhere in the human body where a biocompatible permanent injectable material is necessary. The suspension can be injected endoscopically, for example through a laryngoscope for injection into the vocal chords for the treatment of dysphonia, or through a hysteroscope for injection into the fallopian tubes as a method of rendering the patient infertile, or through a proctoscope, for injection of the substance in the perirectal sphincter area, thereby increasing the resistance in the sphincter area and rendering the patient continent of stool.

This technology can be used for other purposes. For example, custom-molded cell implants can be used to reconstruct three dimensional tissue defects, e.g., molds of human ears could be created and a chondrocyte-hydrogel replica could be fashioned and implanted to reconstruct a missing ear. Cells can also be transplanted in the form of a thee-dimensional structure which could be delivered via injection.

The teachings of the cited publications are indicative of the level of skill and the general knowledge of those skilled in the art. To the extent necessary, the publications are specifically incorporated herein by reference.

I claim:

1. A method for implanting tissue into an animal comprising

mixing dissociated cells with a solution of a biocompatible polymer capable of crosslinking to form a hydrogel;

implanting the mixture into the animal; and

permitting the polymer to crosslink and form a hydrogel matrix having the cells dispersed therein.

2. The method of claim 1 wherein the hydrogel matrix containing the cells forms before implantation.

3. The method of claim 1 wherein the mixture of the polymer and the cells is injected into the animal as a cell suspension; and

wherein the hydrogel matrix containing the cells forms after the mixture is injected.

4. The method of claim 1 wherein the polymer is a mixture of polyethylene oxide and polyacrylic acid.

5. The method of claim 1 wherein the polymer is selected from the group consisting of a modified aliginate, a modified hyaluronic acid and gelatin.

6. The method of claim 1 wherein the hydrogel is formed by exposure of the polymer to a crosslinking agent selected from the group consisting of ions, pH changes, radical initiators, enzymes, and temperature changes.

7. The method of claim 1 wherein the polymer is a polysaccharide which forms a hydrogel upon exposure to a monovalent cation.

8. The method of claim 7 wherein the polysaccharide is selected from the group consisting of a gellan gum and a carrageenan.

9. The method of claim 7 wherein the cation is sodium.

10. The method of claim 6 wherein the polymer comprises substituents capable of crosslinking by a radical reaction in the presence of a radical initiator to form a hydrogel, and wherein the polymer is contacted with the radical initiator thereby to covalently crosslink the polymer by a radical reaction and form the hydrogel.

11. The method of claim 10 wherein the radical initiator is selected from the group consisting of a dye, ultraviolet light and visible light.

12. The method of claim 1 wherein the polymer comprises substituents capable of covalently reacting with a crosslinking agent, and wherein the polymer is contacted with the crosslinking agent thereby to covalently crosslink the polymer and form the hydrogel.

13. The method of claim 1 wherein the cells are selected from the group consisting of chondrocytes and other cells that form cartilage, osteoblasts and other cells that form bone, muscle cells, fibroblasts, and organ cells.

14. The method of claim 2 wherein the hydrogel is molded to form a specific shape prior to implantation.

15. The method of claim 3 wherein the hydrogel is molded to form a specific shape after mixing with the cells and being implanted into the animal.

16. The method of claim 1 for treating vesicoureteral reflux.

17. The method of claim 1 for treating incontinence.

18. The method of claim 1 for treating a defect in the thoracic region.

19. The method of claim 1 for treating the upper gastrointestinal tract.

20. A composition for implanting cells in an animal comprising:

a mixture of dissociated cells and a solution of a biocompatible polymer capable of crosslinking to form a hydrogel matrix having the cells dispersed therein.

21. The composition of claim 20 wherein the polymer is capable of forming the hydrogel matrix having cells disposed therein prior to implantation in the animal.

22. The composition of claim 20 wherein the polymer is capable of forming the hydrogel matrix having cells disposed therein after implantation of the mixture in the animal by injection.

23. The composition of claim 20 wherein the polymer is selected from the group consisting of a modified aliginate, a modified hyaluronic acid and gelatin.

24. The composition of claim 20 wherein the hydrogel is formed by exposure of the polymer to a crosslinking agent selected from the group consisting of ions, pH changes, radical initiators, enzymes, and temperature changes.

25. The composition of claim 20 wherein the polymer is a polysaccharide which forms a hydrogel upon exposure to a monovalent cation.

26. The composition of claim 25 wherein the polysaccharide is selected from the group consisting of a gellan gum and a carrageenan.

27. The composition of claim 25 wherein the cation is sodium.

28. The composition of claim 24 wherein the polymer comprises substituents capable of crosslinking by a radical reaction in the presence of a radical initiator to form a hydrogel.

29. The composition of claim 20 wherein the polymer comprises substituents capable of covalently reacting with a crosslinking agent, thereby to covalently crosslink the polymer and form the hydrogel.

30. The composition of claim 20 wherein the cells are selected from the group consisting of chondrocytes and other cells that form cartilage, osteoblasts and other cells that form bone, muscle cells, fibroblasts, and organ cells.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/09065

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61L27/00 C12N5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61L C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 25080 (MASSACHUSETTS INST TECHNOLOGY ;CHILDRENS MEDICAL CENTER (US)) 10 November 1994 cited in the application see claims ---	1-30
X	US,A,4 846 835 (GRANDE DANIEL A) 11 July 1989 see the whole document ---	1-3,13
P,X	WO,A,95 26761 (COLLAGEN CORP) 12 October 1995 see claims ---	1-3, 20-22
P,X	WO,A,96 03160 (CHILDRENS MEDICAL CENTER ;GEN HOSPITAL CORP (US)) 8 February 1996 see claims --- -/--	1-3,13, 16-19,30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 October 1996	04. 11. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	ESPINOSA, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PC./US 96/09065

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,88 03785 (VACANTI JOSEPH P ;LANGER ROBERT S (US)) 2 June 1988 see claims ---	1-30
A	WO,A,91 01720 (SCHLAMEUS HERMAN WADE ;FOX WILLIAM CASEY (US); MANGOLD DONALD JACO) 21 February 1991 see claims -----	1-30

1

INTERNATIONAL SEARCH REPORT

I national application No.
PCT/US 96/09065

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 1-19
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : although claims 1-19 are directed to a method of treatment of the human or animal body the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr ional Application No
PC,/US 96/09065

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9425080	10-11-94	AU-A-	7015794	21-11-94
		EP-A-	0708662	01-05-96
US-A-4846835	11-07-89	NONE		
WO-A-9526761	12-10-95	AU-A-	2202995	23-10-95
WO-A-9603160	08-02-96	AU-A-	3145795	22-02-96
WO-A-8803785	02-06-88	AT-T-	139432	15-07-96
		DE-D-	3751843	25-07-96
		EP-A-	0299010	18-01-89
		JP-B-	7102130	08-11-95
		JP-T-	1501362	18-05-89
		US-A-	5041138	20-08-91
WO-A-9101720	21-02-91	US-A-	5294446	15-03-94

Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: Implications for cartilage tissue repair

J. Kisiday*, M. Jin†, B. Kurz‡, H. Hung‡, C. Semino§, S. Zhang§¶, and A. J. Grodzinsky*†‡§¶

*Biological Engineering Division, Departments of †Mechanical and ‡Electrical Engineering, §Center for Biomedical Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139-4307

Communicated by John M. Buchanan, Massachusetts Institute of Technology, Cambridge, MA, May 23, 2002 (received for review February 23, 2002)

Emerging medical technologies for effective and lasting repair of articular cartilage include delivery of cells or cell-seeded scaffolds to a defect site to initiate *de novo* tissue regeneration. Biocompatible scaffolds assist in providing a template for cell distribution and extracellular matrix (ECM) accumulation in a three-dimensional geometry. A major challenge in choosing an appropriate scaffold for cartilage repair is the identification of a material that can simultaneously stimulate high rates of cell division and high rates of cell synthesis of phenotypically specific ECM macromolecules until repair evolves into steady-state tissue maintenance. We have devised a self-assembling peptide hydrogel scaffold for cartilage repair and developed a method to encapsulate chondrocytes within the peptide hydrogel. During 4 weeks of culture *in vitro*, chondrocytes seeded within the peptide hydrogel retained their morphology and developed a cartilage-like ECM rich in proteoglycans and type II collagen, indicative of a stable chondrocyte phenotype. Time-dependent accumulation of this ECM was paralleled by increases in material stiffness, indicative of deposition of mechanically functional neo-tissue. Taken together, these results demonstrate the potential of a self-assembling peptide hydrogel as a scaffold for the synthesis and accumulation of a true cartilage-like ECM within a three-dimensional cell culture for cartilage tissue repair.

three-dimensional cell culture | biological scaffold | regenerative medicine

Articular cartilage defects, resulting from traumatic injury or degenerative diseases, may require novel regenerative medicine strategies for restoration of biologically and mechanically functional tissue. One approach relies on delivering cells within voids created by the removal of dysfunctional or damaged tissue (1). Implanted cells within the wound bed may initiate a repair response through *de novo* cellular regulation. This strategy remains a significant challenge in current healthcare technologies. Delivery of chondrocytes to a cartilage defect may be facilitated by attachment to or encapsulation within a scaffold. Tissue engineering scaffolds must be completely biocompatible, without the potential to degrade into harmful residues. The scaffold defines a three-dimensional (3D) template in which chondrocytes produce and deposit extracellular matrix (ECM). Structural stability of the cell/scaffold system must be maintained by the scaffold until seeded chondrocytes have deposited a continuous network of ECM throughout the implant. Ideally, the scaffold would then degrade as the ECM network matures, guiding regeneration throughout the entire scaffold geometry. A successful cartilage replacement must integrate with surrounding normal cartilage, and the newly assembled ECM must provide tissue resilience to tissue compression that occurs during normal joint loading.

A variety of biologically derived and synthetic polymeric and hydrogel materials are actively under investigation as scaffolds for cartilage tissue repair (2). Collagen-based scaffolds [e.g., type I collagen gel (3) and type I and type II collagen sponges (4, 5)], polyglycolic acid and polylactic acid (6), fibrin (7), alginate (8), and polyethlene oxide (9) have been studied *in vitro* to characterize chondrocyte division and phenotypic expression as well as ECM production. Animal studies have also been conducted with these scaffolds, as well as a hyaluronon derivative, to test ECM formation and cell-scaffold integration *in vivo* (6, 10–14). Whereas many scaffolds maintain differentiated chondrocytes and accumulate ECM matrix *in vitro* and/or in animals, no clinical application of a scaffold-based cartilage repair tissue is yet available.

A new class of peptide-based biomaterials has been actively pursued as a molecular-engineered scaffold for tissue repair. Certain peptides are able to self-assemble into stable hydrogels at low (0.1–1%) peptide concentrations (15–17). Such self-assembling peptides are characterized by amino acid sequences of alternating hydrophobic and hydrophilic side groups. Sequences of charged amino acid residues include alternating positive and negative charges (15–17). Self-assembling peptides form stable β-sheet structures when dissolved in deionized water. Exposure to electrolyte solution initiates β-sheet assembly into interweaving nanofibers (15–17). Such self-assembly occurs rapidly when the ionic strength of the peptide solution exceeds a certain threshold, or the pH is such that the net charge of the peptide molecules is near zero (18). Intermediate steps of self-assembly have been investigated by observing relatively slow nanofiber formation and subsequent network assembly in deionized water, without triggering rapid self-assembly by the addition of electrolytes (19). The self-assembling peptide hydrogel contains unique features for a tissue engineering polymer scaffold. The nanofiber structure is almost 3 orders of magnitude smaller than most polymer microfibers and presents a unique polymer structure with which cells may interact. In addition, peptide sequences may be designed for specific cell-matrix interactions that influence cell differentiation and tissue formation (20). Also, the synthetic nature of the peptide minimizes the risk of carrying biological pathogens relative to animal-derived biomaterials (20).

Diverse mammalian cell types have been found to attach and proliferate on the preassembled peptide gel surfaces (16). Additionally, two peptides have been tested for immunogenicity in rats. Injection of $(EAKA)_4$ and $(RADA)_4$ into leg muscle of Fisher 344 rats resulted in no detectable toxic reaction after 9 days and 5 weeks, respectively (17).

Here, we report the study of a self-assembling peptide KLD-12 hydrogel as a 3D scaffold for encapsulation of chondrocytes. Previously, investigators found that chondrocytes could maintain their phenotype or redifferentiate after serial passaging in hydrogels such as agarose (21) and alginate (22). Such model hydrogel culture systems have been useful for studying the basic biology of chondrocyte biosynthesis of ECM (23) and cellular response to mechanical loading (24) *in vitro*. In this study, we hypothesized that a self-assembling peptide hydrogel would provide an appropriate environment for the retention of chondrocyte phenotype and the synthesis of a mechanically functional cartilage ECM. In addition,

Abbreviations: ECM, extracellular matrix; 3D, three-dimensional; ITS, insulin, transferrin, and selenium; GAG, glycosaminoglycan; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

¶To whom reprint requests may be addressed. E-mail: alg@mit.edu or shuguang@mit.edu.

www.pnas.org/cgi/doi/10.1073/pnas.142309999

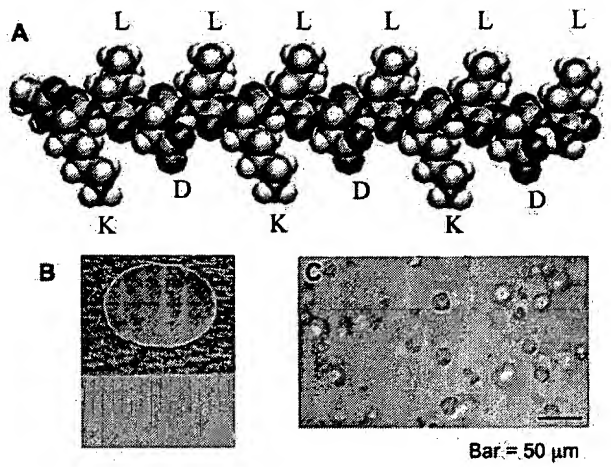

Fig. 1. (*A*) Molecular model of a single KLD-12 self-assembling peptide. The alternating hydrophobic and hydrophilic residues on the backbone promote β-sheet formation. The positively charged lysines (K) and negatively charged aspartic acids (D) are on the lower side of the β-sheet, and the hydrophobic leucines (L) are on the upper side. This molecular structure facilitates self-assembly through intermolecular interactions. (*B*) A 12-mm chondrocyte-seeded peptide hydrogel plug, punched from 1.6-mm-thick slabs. (*C*) Light microscope image of chondrocytes encapsulated in peptide hydrogel.

the self-assembling nature of the peptide hydrogel and the flexibility of molecular design may offer advantages in controlling scaffold degradation, cell attachment, and the delivery of tethered stimulatory growth factors to the microenvironment of encapsulated cells. Together, these features offer a flexible approach to optimizing scaffold-cell interactions for cartilage repair.

Materials and Methods

Isolation of Chondrocytes and Casting of Cell-Seeded Peptide and Agarose Hydrogels. Chondrocytes were isolated from the femoropatellar grooves of 1- to 2-week-old bovine calves within several hours after slaughter, as described (25). The peptide KLD-12 with sequence AcN-KLDLKLDLKLDL-CNH_2 (Fig. 1*A*) was synthesized by using a peptide synthesizer (Applied Biosystems Peptide synthesizer 431A) and lyophilized to a powder. KLD12 powder was dissolved in 295 mM sucrose solution at a peptide concentration of 0.56% (wt/vol). Isolated chondrocytes were resuspended in a volume of sucrose solution equal to 10% of the final peptide/cell suspension volume. Peptide solution was added to obtain a final peptide concentration of 0.5% and a cell density of 15×10^6 cells per ml, with the sucrose used to maintain physiologic osmotic pressure. The suspension was lightly vortexed and injected into a stainless steel casting frame consisting of a $40 \times 40 \times 1.6$-mm window, supported on both faces by filter paper and porous mesh similar to Ragan *et al.* (25). The casting frame was placed in a 1× PBS bath to initiate self-assembly of the peptide gel into a slab structure. After 25 min, the 1.6-mm-thick seeded peptide gel was transferred to a Petri dish for long-term culture.

Chondrocyte-seeded agarose gel slabs were cast in a similar manner. Centrifuged cells were resuspended in feed medium and mixed with 3% low melting temperature agarose (SeaPlaque agarose, FMC) to obtain a final agarose concentration of 2% and a cell density of 15×10^6 cells per ml. The casting frame was placed in a room temperature 1× PBS bath to initiate gelation. After 5 min the gel slab was transferred to a Petri dish containing 4°C medium and subsequently placed in a 37° incubator. Peptide and agarose gels were cultured in 10% FBS-supplemented feed medium. Additional gel preparations were maintained in feed medium supplemented with 1% ITS (insulin, transferrin, and selenium, Sigma) plus 0.2% FBS (ITS/FBS medium). The slabs were seeded at a cell density of 15×10^6 or 30×10^6 cells per ml. Each slab was fed 12 ml of medium every other day.

Cellular Biosynthesis of ECM Macromolecules. On days 5, 10, 15, 21, and 28 of culture, groups of six 3-mm diameter by 1.6-mm thick plugs were punched from cell-seeded peptide and agarose gel slabs by using a stainless steel dermal punch. Each group of plugs was transferred to a single well of a 12-well dish containing 2 ml feed medium plus 5 μCi/ml ^{35}S-sulfate and 10 μCi/ml ^{3}H-proline. Incorporation of ^{35}S-sulfate and ^{3}H-proline are measures of the rate of synthesis of sulfated proteoglycans and total protein, respectively (26). After 20 h the plugs were removed from radiolabel medium and washed five times over 90 min in standard PBS plus 1 mM unlabeled proline and sulfate. Each cell-seeded plug was then transferred to 1-ml proteinase K (Roche Applied Science)–Tris·HCl solution and digested overnight at 60°C. From the digests, radiolabel incorporation and total accumulated sulfated glycosaminoglycan (GAG) content via DMMB dye binding were measured as described (27).

Separate control studies on day 35 of culture were performed to assess (*i*) the fraction of the radioactivity within the peptide hydrogel plugs that was in macromolecular versus low molecular weight form, (*ii*) the fraction of total newly synthesized ECM macromolecules that was retained within the peptide hydrogel versus that released to medium, and (*iii*) the fraction of ^{3}H radiolabel incorporated as hydroxyproline as a measure of newly synthesized collagen. For macromolecular analysis, radiolabeled species obtained from peptide gel extracts and medium fractions were separated into macromolecular and low molecular weight components on a PD10 gel filtration column of Sephadex G-25 (molecular weight cutoff of 1–5,000; Amersham Pharmacia) in 0.5-ml portions of elution buffer containing 1% SDS, 10 mM DTT, 50 mM Tris·HCl, pH 8.5 (21). Macromolecular components released to medium were ≈2% and ≈11% of total peptide hydrogel-accumulated ^{35}S-sulfate and ^{3}H-proline, respectively. Proteinase K-digested peptide gel plugs contained 95% macromolecular ^{35}S-sulfate. Hydrogel-accumulated ^{3}H-proline was analyzed after SDS extraction. Cell-seeded peptide hydrogel was incubated in 2% SDS, 10 mM DTT, 50 mM Tris·HCl, pH 8.5 at 100°C for 15 min. Extracted ^{3}H-proline molecules were 89% macromolecular. These data showed that the hydrogel scaffold retained ≈93% and 78% of newly synthesized proteoglycan and protein macromolecules, respectively, similar to that observed in native cartilage organ culture (27). Because of the high retention of macromolecules in the hydrogel scaffold radiolabel medium was not routinely analyzed for biosynthetic products. Peptide gel extracts were analyzed for ^{3}H-hydroxyproline content (28). The ratio of incorporated (^{3}H-hydroxyproline)/(^{3}H-proline) was multiplied by 2.2 to calculate the percent newly synthesized collagen, based on the 1:1.2 molar ratio of hydroxyproline to proline in bovine type II collagen (29). Thus, newly synthesized proteins were determined to be ≈70% collagen. These data demonstrate that the percentage of newly synthesized proteins found as collagen is similar to that observed in native calf cartilage organ cultures of the same age as the cells used in this study (30).

Histological Examination of Chondrocytes and Newly Synthesized ECM Within Peptide Hydrogels. Cell-seeded peptide specimens were fixed in 4% paraformalydehyde in PBS overnight at 4°C, washed, dehydrated with isopropanol, transferred to xylol, and embedded in paraffin. Sections 7 μm thick were made and spread on slides coated with chromealaun-gelatin (31), deparaffinated in xylol, and transferred into aqua dest by using decreasing concentrations of ethanol. Some sections were incubated for 6 min with toluidine blue dye solution [0.0714% toluidine blue (Merck), 0.0714% pyronin Y (Fluka), and borax (0.143% di-sodium-tetra-borate, Merck)],

washed subsequently with aqua dest, 96% ethanol, propanol, and xylol, and mounted on microscopic slides with DePeX (Serva).

Collagen Immunohistochemistry. Sections were treated with pepsin for 30 min, washed with TBS buffer, treated with 0.6% H_2O_2 in methanol, rinsed again with TBS, and treated with mouse anticollagen type II IgG (clone CII C1, Developmental Studies Hybridoma Bank, Iowa City, IA, 1:1,000 in TBS) for 60 min, as described (31). After incubation with rabbit anti-mouse IgG (horseradish peroxidase-conjugated Dako P-0260; 1:200 in TBS containing 1% bovine serum) for 30 min, the sections were rinsed and incubated for 30 min with goat anti-rabbit IgG (horseradish peroxidase-conjugated Dako P-0448; 1:100 in TBS containing 1% bovine serum). The samples were stained with diaminobenzidine (DAB Kit, Vector Laboratories). Cell nuclei were counterstained with Meyer's hemalum. Stained samples were embedded on microscopic slides with Aquatex (Merck).

Collagen Isolation and SDS/PAGE. Selected 3-mm cell-seeded peptide plugs (initial seeding density 15×10^6 cells per ml) cultured in ITS/FBS medium were punched on day 35 and lyophilized. Collagens were extracted with pepsin (Sigma, 1 mg/ml in 0.2 M NaCl, 0.5 M acetic acid) as described (32), except 4.5 M NaCl was used to precipitate collagen rather than ammonium sulfate. Collagens extracted from chick cartilage and mouse skin (a generous gift from P. Bruckner, University of Münster, Münster, Germany) were run in parallel to the cell-seeded peptide extract as standards for type II collagen synthesized by native chondrocytes and type I collagen synthesized by dedifferentiated fibroblastic phenotypes, respectively (P. Bruckner, personal communication).

Mechanical Properties of Cell-Seeded Hydrogels. Groups of cell-seeded peptide plugs were punched on days 0, 6, and 26 from the 10% FBS culture and day 28 of the ITS/FBS culture. The equilibrium modulus and dynamic stiffness of each plug was measured in radially confined uniaxial compression by using a Dynastat mechanical spectrometer (IMASS, Hingham, MA) as described (33). Briefly, individual specimens were placed in a confining cylindrical chamber that was clamped in the jaws of the Dynastat. A porous platen attached to the upper jaw was used to apply 4–6 sequential ramp-and-hold compressive strains of 3% to the plug (3% compression over 10 sec followed by a 1–5 min of hold), resulting in an initial increase and subsequent relaxation of the compressive stress. The ratio of the relaxed equilibrium stress to the engineering strain was used to compute the equilibrium modulus. At 18% compressive offset strain, a 1% amplitude sinusoidal strain was applied at 1.0 Hz. The dynamic compressive stiffness was calculated as the ratio of the fundamental amplitudes of stress to strain (33).

Chondrocyte Division Within Peptide Hydrogel. Peptide in digested samples was found to cause interference with the DNA spectrofluorometric measurement of Kim *et al.* (34); therefore, cell density measurements in chondrocyte-seeded peptide could not be inferred with this method. A viable cell kit based on the compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega) was used to determine viable cell density in seeded peptide scaffolds. To establish a calibration curve for 3D cultures, five agarose slabs were seeded at cell densities ranging between 10 and 30×10^6 cells per ml. Samples were punched and analyzed on days 2, 3, and 5. For each group of 11 plugs, six were incubated in MTS medium for 2 h on a shaker table. SDS was added (final concentration = 2%) to stop the MTS reaction. The absorbance of medium samples at 490 nm was measured after 30 min. The remaining five plugs were digested and analyzed for DNA content. Mean MTS output was plotted against mean DNA content to establish the calibration curve. MTS data were then obtained for cell-seeded peptide plugs by using the established method.

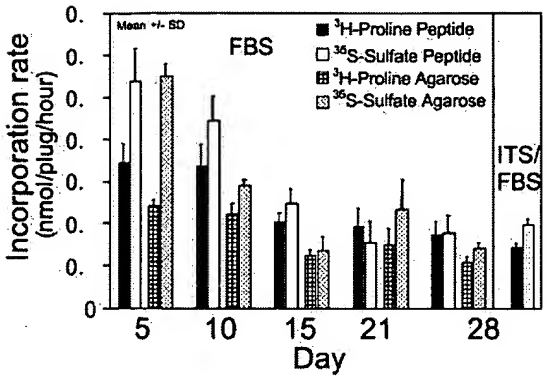

Fig. 2. Radiolabel incorporation of ^{3}H-proline and ^{35}S-sulfate as measures of the synthesis of proteins and sulfated proteoglycans, respectively, by chondrocytes within cell-seeded peptide and agarose hydrogels.

To further verify MTS results, cell-seeded peptide samples were analyzed via 5′ BrdUrd incorporation for 24 h on days 3 and 7. BrdUrd (10 μM) was added to the culture media for 24 h. Samples were fixed in 2% paraformaldehyde in PBS (pH 7.4) at room temperature, washed with PBS, and subsequently treated with 0.1% Triton X-100 in PBS for 2 h, then they were treated with 2 M HCl in PBS for 30 min at 37°C and washed with PBS. Blocking buffer (20% bovine calf serum/0.1% Triton X-100/1% DMSO in PBS) was added to the samples. An anti-BrdUrd antibody IgG_1 FITC-conjugated (PharMingen, 33284X) was used to visualize BrdUrd-positive cells under a Nikon TE 300 inverted microscope with phase contrast and fluorescence.

Results

Casting of Cell-Seeded Peptide Hydrogels. Previously, cell-free peptide hydrogels were formed by first dissolving the peptide in deionized water and then exposing the peptide solution to salt solution or culture medium to initiate self-assembly. Here, 295 mM sucrose solution was used to solubilize the KLD-12 peptide to increase osmolarity to physiological levels and thereby maintain cell viability before addition of electrolyte. Sucrose solution was found to be a suitable medium for peptide dissolution and subsequent self-assembly. Upon immersion in PBS, the casting frame produced flat slabs of chondrocyte-seeded peptide gel from which cylindrical plug samples could be cored (Fig. 1*B*). Encapsulated chondrocytes showed a round morphology within the peptide gel (Fig. 1*C*), similar to that seen in standard agarose or explant cultures. Cell viability in the peptide gel was ≈80% immediately after casting, whereas the same chondrocyte population seeded into parallel agarose culture had ≈95% viability.

Biosynthesis of ECM Macromolecules by Encapsulated Chondrocytes Within Hydrogels. Proteoglycan (^{35}S-sulfate incorporation) and protein (^{3}H-proline) synthesis rates by cells in peptide hydrogels with 10% FBS medium were initially high at early times (day 5) and then decreased by day 15 to values on the order of that in native cartilage tissue (Fig. 2) (23). Subsequent incorporation through day 28 remained relatively constant. Radioisotope incorporation in cell-seeded agarose hydrogels (Fig. 2) revealed biosynthesis levels similar to those in peptide hydrogels and comparable to values previously reported in the literature for agarose culture with bovine calf chondrocytes (23). It is important to emphasize that cell-seeded peptide plugs cultured in minimal medium (ITS/FBS) and radiolabeled on day 28 showed biosynthesis rates similar to those in agarose and peptide hydrogels with 10% FBS-supplemented rich medium.

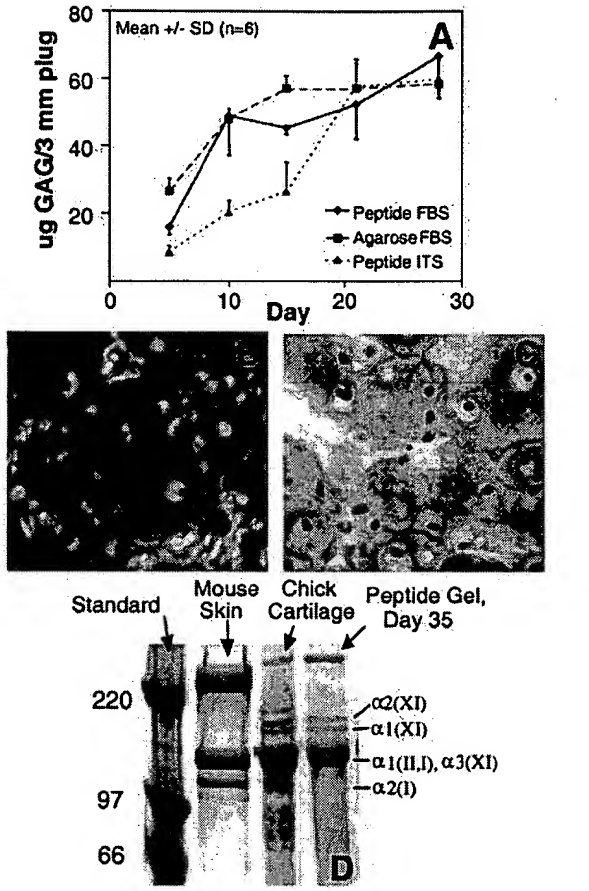

Fig. 3. Matrix accumulation in chondrocyte-seeded peptide hydrogel. (*A*) Total GAG accumulation in cell-seeded peptide hydrogel cultured in FBS and ITS/FBS medium and in cell-seeded agarose. (*B*) Toluidine blue staining of chondrocyte-seeded peptide hydrogel cultured in 10% FBS, day 15. (*C*) Immunohistochemical staining for type II collagen in cell-seeded peptide hydrogel cultured in 10% FBS, day 15. Image width for *B* and *C* = 175 μm. (*D*) SDS/PAGE of collagens extracted from day 35 samples of chondrocyte-seeded peptide hydrogel cultured in 1% ITS with 0.2% FBS. Standards: Chick cartilage for collagen II and XI banding pattern. Mouse skin identifies collagen I α-helix 2, indicative of collagen expression of a dedifferentiated, fibroblastic phenotype.

GAG Accumulation Within Gel-Encapsulated ECM. Total GAG accumulation in peptide hydrogels with 10% FBS medium increased with time in culture, reaching ≈70 μg/plug by day 28 (Fig. 3*A*). Agarose cultures showed similar GAG accumulation, consistent with previous data for agarose culture (23). In ITS/FBS medium, GAG accumulation in peptide gels was initially lower than that in both agarose culture and 10% FBS peptide hydrogel cultures through day 15; however, by days 21 and 28, GAG accumulation was similar in all three cases. Toluidine blue staining of peptide hydrogels cultured in FBS medium revealed GAG accumulation throughout the peptide hydrogel matrix on day 26 (Fig. 3*B*). The majority of the chondrocytes showed a rounded morphology and were fully encapsulated within a continuous GAG-rich matrix, with a higher pericellular staining relative to the interterritorial matrix.

Chondrocyte Phenotypic Expression. Cell-seeded peptide gels cultured in FBS medium were fixed on day 15 for collagen II immunostaining. Collagen II (Fig. 3*C*) showed a staining pattern similar to that of GAG deposition, with positive staining throughout the matrix, especially in pericellular environments. Collagens from

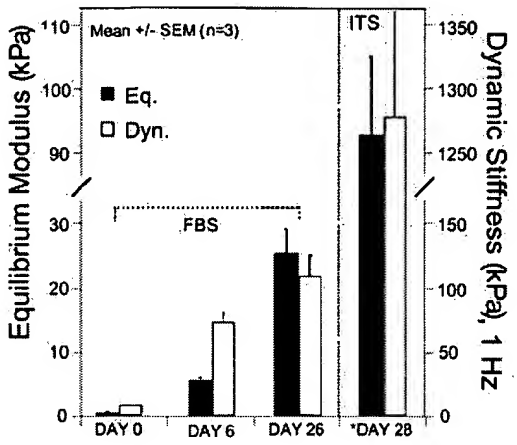

Fig. 4. Mechanical properties of chondrocyte-seeded peptide hydrogels. Equilibrium modulus and dynamic stiffness measured in uniaxial confined compression, evaluated in 6-mm diameter plugs initially seeded at a density of 15×10^6 cells per ml and cultured in 10% FBS medium. * indicates 3-mm diameter plugs seeded at 30×10^6 cells per ml and cultured in ITS/FBS medium, conditions that resulted in markedly increased stiffness values, as high as 20–33% that of human and animal cartilages (26).

cell-seeded peptide hydrogels cultured in ITS medium were extracted on day 35 and analyzed by using SDS gel electrophoresis (Fig. 3*D*). Proteins extracted from cell-seeded peptide hydrogel showed bands for collagen type II α1 chains, as well as α1 and 2 chains for type XI collagen. No type I α 2 chains were present, as seen in skin collagen. Western analysis further confirmed electrophoresis findings. These results demonstrate unambiguously that cells seeded into peptide hydrogels maintained their chondrocyte phenotype throughout the 4-week *in vitro* culture period.

Mechanical Properties of Chondrocyte-Seeded Hydrogels. Day 0 values for equilibrium modulus were less than 1 kPa, which corresponds to the weak compressive resistance of the acellular peptide hydrogel itself (Fig. 4). Development of a continuous GAG matrix was reflected in a dramatic increase in mechanical properties over time in culture. By day 26, the equilibrium modulus increased to 26 kPa, a value about 10% of the modulus of native tissue (35). Dynamic stiffness at 1 Hz also increased with time in culture, with day 26 values about 5% of those of native tissue (35). Interestingly, cells seeded into peptide hydrogels at 30×10^6 cells per ml and cultured in ITS/FBS medium showed an even more profound increase in compressive stiffness. The equilibrium modulus reached 93 kPa by day 28, and the dynamic stiffness at 1 Hz reached 1.28 MPa (Fig. 4), both values being ≈1/5 to 1/3 that of native human and animal articular cartilages (36).

Encapsulated Cell Division. Calibration of the MTS viable cell assay in cell-seeded agarose plugs showed linear behavior over a range of cell densities (Fig. 5*A*). Cell-seeded peptide hydrogels and agarose gels cultured in FBS medium were evaluated for viable cell density by using the MTS assay (Fig. 5*B*). In agarose gels, viable cell density increased by 20% between day 2 and day 7 of culture, an increase similar to that previously reported with DNA (Hoechst dye) analysis (23). In contrast, peptide hydrogels showed a more dramatic 80% increase in viable cell density between days 2 and 9 of culture. BrdUrd incorporation in cell-seeded peptide hydrogels further delineated MTS results (Fig. 5 *C* and *D*). A significant fraction of cells within the peptide hydrogel labeled positively for BrdUrd incorporation during the 24-h incubation period on days 2–3 and 7–8, further indicating a significant content of dividing cells in these cultures.

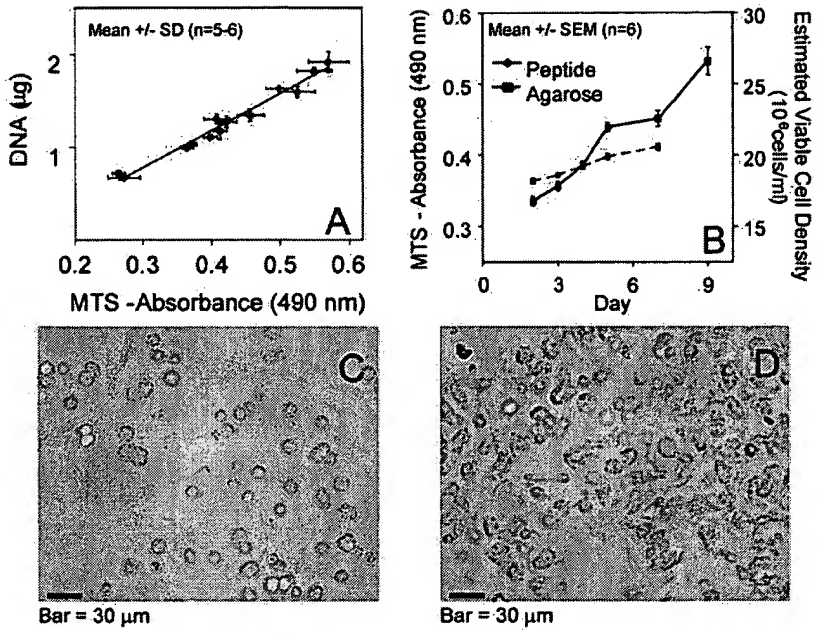

Fig. 5. Cell division of chondrocytes seeded in peptide hydrogel. (*A*) Calibration curve for the MTS assay in chondrocyte-seeded agarose hydrogel. (*B*) MTS measurement of the density of cells in chondrocyte-seeded peptide and agarose hydrogels. (*C* and *D*) BrdUrd incorporation in chondrocyte-seeded hydrogel 3 days (*C*) and 7 days (*D*) after seeding.

Discussion

Casting of Cell-Seeded Peptide Hydrogels. The casting procedures developed here enabled rapid and efficient encapsulation of chondrocytes within peptide hydrogels while maintaining high cell viability (>80%). Encapsulated cells were evenly distributed throughout the hydrogel. In this study, cell-seeded hydrogels were cast in a flat slab geometry to enable quantification of biomechanical properties and normalization of ECM biosynthesis and accumulation to a constant plug volume. However, this casting technique is generally applicable for scaffolds of arbitrary geometry and other cell types, a very important feature for applications in tissue repair. Here, peptides self-assemble into well-ordered nanofibers with inter-fiber spaces ≈50–200 nm and, simultaneously, into a nanoscale hydrogel network (15–17) around each individual chondrocyte in the cell-peptide solution. This intimate cell-scaffold architecture may offer unique advantages regarding peptide-cell signaling and cell-mediated peptide biodegradability.

Chondrocyte Appearance and Division Within the Peptide Hydrogel. Chondrocyte-seeded constructs for cartilage repair may benefit from cell division after seeding in the scaffold to further increase the number of ECM-producing cells. The peptide hydrogel matrix appeared favorable for cell division while maintaining synthesis of phenotypically specific ECM macromolecules. Division of encapsulated chondrocytes was substantially higher in peptide gels relative to agarose control cultures. Abundant cell pairs undergoing cell division were observed within the hydrogel as early as day 3 in culture (Fig. 5 *B–D*). It is not known whether higher cell division in peptide hydrogel relative to agarose can be attributed to cell-peptide interactions, physical environment, or other factors. Ongoing studies exploring specific cell-peptide interactions may give insights into methods for further stimulation of cell division.

Synthesis and Accumulation of ECM Within Peptide Hydrogels. Radiolabel incorporation and total GAG accumulation (Figs. 2 and 3*A*) were normalized on a per-plug basis, as punched from parent slab gels. In this manner, biosynthesis is reported as representative repair tissue generation for a given initial scaffold geometry (e.g., a cylindrical disk specimen) and cell-seeding density. Radiolabel incorporation (Fig. 2) showed that synthesis of proteoglycans and total protein by primary calf chondrocytes was similar in 3D agarose and 3D peptide hydrogel culture. Thus, specific differences in hydrogel composition and microphysical environment between peptide and agarose scaffolds did not significantly affect the net rates of cell synthesis of ECM macromolecules.

Total GAG accumulation in cell-seeded peptide scaffold was also similar to that in agarose culture (Fig. 3*A*), despite the differences in scaffold concentration (0.5% vs. 2% wt/wt). For comparison with other studies in the literature, data from day 15 (Fig. 3*A*) were normalized to DNA content 7.7 pg DNA/cell (34) based on an estimate of the cell density at day 15 (assumed to be ≈2× the initial seeding density from the trends of Fig. 5*B*). Thus, the computed GAG density on day 15 was ≈22 μg/μg DNA. This value is less than that reported in alginate culture (≈50 μg/μg) (25) but greater than that in type I collagen sponge (4.8 μg/μg) (37), polyglycolic acid (≈8 μg/μg) and polylactic acid (≈5 μg/μg) (6). In addition, the percent GAG normalized to wet weight in peptide scaffold (estimate ≈0.9%, week 4) compared with polyethylene oxide, ≈0.23%, week 6, 50 × 10^6 cells per ml initial seeding density (9). Although direct comparisons are difficult because of inconsistent factors such as seeding density and culture medium, GAG accumulation in our peptide hydrogel scaffold appears to be within the range of other polymer and hydrogel scaffolds.

The majority of our experiments used a seeding density of 15 × 10^6 cells per ml in culture with 10% FBS. Such conditions are convenient for comparison to previously published studies but are not necessarily optimal for *in vitro* development of repair tissue. Limited studies using different medium and cell densities were also conducted to demonstrate options for *in vitro* culture conditions. ITS-based, low serum medium (0.2% FBS) was found to be comparable to 10% FBS + DMEM in terms of total GAG accumulation and radioisotope incorporation (on day 28, Fig. 2). However, a 2-fold increase in cell seeding density in combination with culture in ITS/FBS medium resulted in a 4-fold increase in equilibrium compressive modulus and >10-fold increase in dynamic stiffness, much more than would be expected from a proportional increase in seeding density alone. Such variables therefore should be explored in detail to further optimize a chondrocyte/peptide-hydrogel system for clinical use in cartilage repair.

Biomechanical Properties. GAG accumulation in seeded peptide scaffold was distributed throughout the hydrogel, in both cell-

associated and interterritorial space (Fig. 3*B*). The development of continuous ECM was reflected in the increase in compressive stiffness as a function of time in culture (Fig. 4). On day 0 the peptide hydrogel-seeded with 15×10^6 cells per ml was very fragile, with extremely low compressive stiffness (100–1,000 times lower than native cartilage). After 6 days in culture, the handling properties of the plugs were noticeably improved, with equilibrium modulus increasing by almost an order of magnitude. By day 26 the test samples could be freely handled and attained an equilibrium modulus of 26 kPa (approximately 30-fold increase compared with day 0). For comparison, the equilibrium modulus of polyglycolic acid scaffolds seeded with primary calf chondrocytes at $\approx 125 \times 10^6$ cells per ml and cultured in free-swelling conditions was ≈52 kPa after 42 days (38), and the modulus of peptide hydrogel scaffolds with 30×10^6 cells per ml in ITS/FBS was 93 kPa (Fig. 4).

Immunohistochemical analysis for type II collagen showed positive staining throughout the peptide hydrogel in a continuous manner, whereas type I collagen staining appeared at only background levels around cells within the gel. With 10% FBS-supplemented medium, positive type I collagen staining was observed within a thin layer of fibroblastic-like cells that showed increasing cell numbers with time in culture only on the outer surfaces of the gel. Interestingly, cell-seeded peptide hydrogels cultured in ITS/FBS medium were observed to have much less dedifferentiation and fibroblast-like cell accumulation on the surface. Therefore, cell samples were chosen from ITS/FBS culture for collagen content analysis. SDS-gel electrophoresis confirmed accumulation of predominantly type II collagen in the cell-seeded peptide hydrogel culture (Fig. 3*D*).

An Emerging Biomaterial for Regenerative Medicine. Our study demonstrates the potential of a self-assembling peptide scaffold to maintain differentiated chondrocytes and stimulate the synthesis and accumulation of a mechanically functional cartilage-like ECM in a 3D cell culture. The peptide KLD12 used in this study represents one of a class of specially designed self-assembling peptides developed through molecular engineering. The synthetic nature of the material allows for single amino acid design, with sequences tailored for cell signaling and/or scaffold degradation. Self-assembly of this class of peptides occurs at physiological electrolyte concentrations and pH, without toxic processing procedures. However, each peptide must first be carefully screened *in vivo* to ensure the sequence does not elicit an immunogenic response by the host animal.

The optimal peptide hydrogel scaffold for successful cartilage tissue repair must enable long-term tissue formation and integration with surrounding tissue *in vivo*. Our ongoing studies are aimed at optimization of cartilage ECM development within the peptide for *in vivo* implantation. ECM synthesis may be further stimulated by addition of growth factors (39) and culture in bioreactors that increase nutrient transport (5, 37, 38) and apply mechanical loads (40, 41). The interaction between chondrocyte and peptide matrix is being investigated by designing binding sites in the peptide sequence. Furthermore, peptides can be designed to include specific sites to enable enzymatic degradation by matrix metalloproteinases and aggrecanases that are naturally synthesized by chondrocytes. In this manner, spatial and temporal control of scaffold biodegradability may be obtained. In addition, the synthetic manufacturing of peptides may allow for tethering of growth factors to subpopulations of peptide units, enabling controlled concentrations of growth factors to be directly delivered to encapsulated cells. Such flexibility offers a broad spectrum of opportunities to investigate multiple approaches toward generating the appropriate tissue repair response. Finally, previous studies have shown that dynamic mechanical compression (27) and shear (42) loading of cartilage explants can dramatically stimulate proteoglycan and collagen synthesis relative to unloaded control cultures. Therefore, mechanical loading of self-assembling peptide gel-encapsulated chondrocytes may stimulate development of an ECM that is rich in proteins or proteoglycans relative to standard free-swelling culture. Such strategies may be useful in developing unique ECM components in cell-seeded peptide scaffolds for repairing cartilage defects in the body.

We thank Michael Caplan, Alex Schermer, and Tom Crowell for their technical consultation. This work was supported in part by grants from the National Institutes of Health (Grant AR33236) and the DuPont–Massachusetts Institute of Technology Alliance.

1. Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O. & Peterson, L. (1994) *N. Engl. J. Med.* **331**, 889–895.
2. Glowacki, J. (2000) *J. Rehabil. Res. Dev.* **37**, 171–177.
3. Schuman, L., Buma, P., Versleyen, D., de Man, B., van der Kraan, P. M., van den Berg, W. B. & Homminga, G. N. (1995) *Biomaterials* **16**, 809–814.
4. Nehrer, S., Breinan, H. A., Ramappa, A., Shortkroff, S., Young, G., Minas. T., Sledge, C. B., Yannas, I. V. & Spector, M. (1997) *J. Biomed. Mater. Res.* **38**, 95–104.
5. Grande, D. A., Halberstadt, C., Naughton, G., Schwartz, R. & Manji, R. (1997) *J. Biomed. Mater. Res.* **34**, 211–220.
6. Freed, L. E., Marquis, J. C., Nohria, A., Emmanual, J., Mikos, A. G. & Langer, R. (1993) *J. Biomed. Mater. Res.* **27**, 11–23.
7. Perka, C., Spitzer, R. S., Lindenhayn, K., Sittinger, M. & Schultz. O. (2000) *J. Biomed. Mater. Res.* **49**, 305–311.
8. Hauselmann, H. J., Fernandes, R. J., Mok, S. S., Schmid, T. M., Block, J. A., Aydelotte, M. B., Kuettner, K. E. & Thonar, E. J. (1994) *J. Cell Sci.* **107**, 17–27.
9. Bryant, S. J. & Anseth, K. S. (2001) *Biomaterials* **22**, 619–626.
10. Wakitani, S., Goto, T., Young, R. G., Mansour, J. M., Goldberg, V. M. & Caplan, A. I. (1998) *Tissue Eng.* **4**, 429–444.
11. Nehrer, S., Breinan, H. A., Ramappa, A., Hsu, H. P., Minas, T., Shortkroff, S., Sledge, C. B., Yannas, I. V. & Spector, M. (1998) *Biomaterials* **19**, 2313–2328.
12. Fragonas, E., Valente, M., Pozzi-Mucelli, M., Toffanin, R., Rizzo, R., Silvestri, F. & Vittur, F. (2000) *Biomaterials* **21**, 795–801.
13. Elisseeff, J., Anseth, K., Sims, D., McIntosh, W., Randolph, M. & Langer, R. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 3104–3107.
14. Grigolo, B., Roseti, L., Fiorini, M., Fini, M., Giavaresi, G., Aldini, N. N., Giardino, R. & Facchini A. (2001) *Biomaterials* **22**, 2417–2424.
15. Zhang, S., Holmes, T., Lockshin, C. & Rich, A. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3334–3338.
16. Zhang, S., Holmes, T. C., DiPersio, C. M., Hynes, R. O., Su, X. & Rich, A. (1995) *Biomaterials* **16**, 1385–1393.
17. Holmes, T. C., de Lacalle, S., Su, X., Liu, G., Rich, A. & Zhang, S. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 6728–6833.
18. Caplan, M. R., Moore, P. N., Zhang, S., Kamm, R. D. & Lauffenburger, D. A. (2000) *Biomacromolecules* **1**, 627–631.
19. Marini, D. M., Hwang, W., Lauffenburger, D. A., Zhang, S. & Kamm, R. D. (2002) *Nanoletters* **2**, 295–259.
20. Holmes, T. C. (2002) *Trends Biotechnol.* **20**, 16–21.
21. Benya, P. D. & Shaffer, J. D. (1982) *Cell* **30**, 215–224.
22. Lemare, F., Steimberg, N., Le Griel, C., Demignot, S. & Adolphe, M. (1998) *J. Cell. Physiol.* **176**, 303–313.
23. Buschmann, M. D., Gluzband, Y. A., Grodzinsky, A. J., Kimura, J. H. & Hunziker, E. B. (1992) *J. Orthop. Res.* **10**, 745–758.
24. Buschmann, M. D., Gluzband, Y. A., Grodzinsky, A. J. & Hunziker, E. B. (1995) *J. Cell Sci.* **108**, 1497–1508.
25. Ragan, P. M., Chin, V. I., Hung, H. H., Masuda, K., Thonar, E. J., Arner, E. C., Grodzinsky, A. J. & Sandy, J. D. (2000) *Arch. Biochem. Biophys.* **383**, 256–264.
26. Hascall, V. C., Handley, C. J., McQuillan, D. J., Hascall, G. K., Robinson, H. C. & Lowther, D. A. (1983) *Arch. Biochem. Biophys.* **224**, 206–223.
27. Sah, R. L., Kim, Y. J., Doong, J. Y., Grodzinsky, A. J., Plaas, A. H. & Sandy, J. D. (1989) *J. Orthop. Res.* **7**, 619–623.
28. Stern, B. D., Mechanic, G. L. & Glimcher, M. J. (1963) *Biochem. Biophys. Res. Commun.* **13**, 137–143.
29. Herbage, D., Bouillet, J. & Bernengo, J.-C. (1977) *Biochem. J.* **161**, 303–312.
30. Sah, R. L., Doong, J. Y., Grodzinsky, A. J., Plass, A. H. K. & Sandy, J. D. (1991) *Arch. Biochem. Biophys.* **286**, 20–29.
31. Domm, C., Schünke, M., Christesen, K. & Kurz, B. (2002) *Osteoarthritis Cart.* **10**, 13–22.
32. Bruckner, P., Horler, I., Mendler, M., Houze, Y., Winterhalter, K. H., Eich-Bender, S. F. & Spycher, M. A. (1989) *J. Cell Biol.* **109**, 2537–2545.
33. Frank, E. H. & Grodzinsky, A. J. (1987) *J. Biomech.* **20**, 615–627.
34. Kim, Y. J., Sah, R. L., Doong, J. Y. & Grodzinsky, A. J. (1988) *Anal. Biochem.* **174**, 168–176.
35. Eisenberg, S. R. & Grodzinsky, A. J. (1985) *J. Orthop. Res.* **3**, 148–159.
36. Chen, A. C., Bae, W. C., Schinagl, R. M. & Sah, R. L. (2001) *J. Biomech.* **34**, 1–12.
37. Mizuno, S., Allemann, F. & Glowacki, J. (2001) *J. Biomed. Mater. Res.* **56**, 368–375.
38. Vunjak-Novakovic, G., Martin, I., Obradovic, B., Treppo, S., Grodzinsky, A. J., Langer, R. & Freed, L. E. (1999) *J. Orthop. Res.* **17**, 130–138.
39. van Osch, G. J., van den Berg, W. B., Hunziker, E. B. & Hauselmann, H. J. (1998) *Osteoarthritis Cart.* **6**, 187–195.
40. Frank, E. H., Jin, M., Loening, A. M., Levenston, M. E. & Grodzinsky, A. J. (2000) *J. Biomech.* **33**, 1523–1527.
41. Mauck, R. L., Soltz, M. A., Wang, C. C., Wong, D. D., Chao, P. H., Valhmu, W. B., Hung, C. T. & Ateshian, G. A. (2000) *J. Biomech. Eng.* **122**, 252–260.
42. Jin, M., Frank, E. H., Quinn, T., Hunziker, E. B. & Grodzinsky, A. J. (2001) *Arch. Biochem. Biophys.* **395**, 41–48.

ELSEVIER

Materials as morphogenetic guides in tissue engineering

Jeffrey A Hubbell

Within native tissues cells are held within the extracellular matrix (ECM), which has a role in maintaining homeostasis, guiding development and directing regeneration. Efforts in tissue engineering have aimed to mimick the ECM to help guide morphogenesis and tissue repair. Studies have not only looked at ways to mimick the structure and characteristics of the ECM, but have also considered ways to reproduce its molecular properties including its bioadhesive character, proteolytic susceptibility and ability to bind growth factors.

Addresses
Institute for Biological and Chemical Engineering, Swiss Federal Institute of Technology Lausanne (EPFL), CH-1015 Ecublens, Switzerland
e-mail: jeffrey.hubbell@epfl.ch

Current Opinion in Biotechnology 2003, 14:551–558

This review comes from a themed issue on
Tissue and cell engineering
Edited by Jeffrey Hubbell

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2003.09.004

Abbreviations

BMP	bone morphogenetic protein
ECM	extracellular matrix
GAG	glycosaminoglycan
MMP	matrix metalloproteinase

Introduction

Cells in native tissues exist within a three-dimensional, viscoelastic milieu rich with biological information, the extracellular matrix (ECM), and interactions with this matrix play an important role in guiding development, maintaining homeostasis and directing regeneration. Considerable effort has been expended in mimicking the ECM to guide morphogenesis in tissue repair and tissue engineering. These efforts in biomimicry have been directed towards mimicking the biochemical composition of the ECM, its fibrillar structure, and its viscoelastic gel characteristics. Recently, efforts under way in several laboratories have been directed towards mimicry at the molecular level of the ECM's bioadhesive character, proteolytic susceptibility, and growth factor binding capacity. To one extent or another, the characteristics of the natural ECM have been partially reconstituted with bioderived materials or replaced with biosynthetic or synthetic materials. Features of particular interest include how the material is formed and how cells come into intimate contact with the matrix, what biological signals they can present to and receive from cells and the extent to which they can respond to cell-derived signals.

This review presents these materials as three different classes. First, we consider the use of biologically derived materials to reconstitute only limited aspects of the ECM. Second, the use of biosynthetic and synthetic materials to reconstitute certain mechanical aspects of the ECM is discussed. Finally, we look at the design of synthetic materials to mimic the bi-directional biomolecular interactions that naturally occur between cells and the ECM surrounding them, which guide tissue remodeling and maintain tissue homeostasis.

The use of biological molecules in partial reconstitution of the ECM

Given the ability to purify components of the ECM, to isolate them from microbial cultures and to produce them in recombinant cells, the reconstitution of certain characteristics of the ECM through the use of selected biomolecular components of the ECM has captured the attention of several research groups.

Collagen

Collagen can be readily purified from animal tissues, such as skin and tendon, as well as human tissues, such as placenta, and reconstituted into gels by changing the pH and temperature of suspensions of the precursor components. These materials have been utilized extensively in studies of the biophysics of cell motility [1,2••] and contraction [1,3], for example. Cell migration in these materials can proceed through mechanisms that either require the action of matrix metalloproteinases (MMPs) or are independent of MMPs [2••]. This potential MMP independence of cell migration arises from the non-cross-linked character of reconstituted collagen, as well as the relatively large mesh size of the collagen network, which is on the length scale of cellular processes. Indeed, cell tracks have been observed in three-dimensional collagen gels behind cells in migration in culture, and these tunnels have been observed to close over time [2••]. When collagen gels are not constrained at their edges, most cells within such three-dimensional materials will contract the gel substantially, owing to cell-generated forces [3–5] in an α-smooth muscle actin-mediated manner [6].

To obtain collagenous materials for use in *in situ* tissue repair and regeneration, in the form of matrices that possess sufficient mechanical strength to at least partially resist cell-induced matrix contraction, chemical glycation procedures have been developed to adjust, in a rather controllable manner, the elastic character of collagen gels

[7]. Heat and chemical treatments have also been developed to produce cross-linked collagen sponges. Such materials have been used in bone [8] and cartilage [9] repair.

It is possible to utilize the dipole moment of fibrillar collagen to obtain permanent microscopic and macroscopic alignment of fibrillar collagen matrices under a strong magnetic field [10•]. These aligned collagen matrices have been demonstrated to possess special characteristics for inducing directed cell migration, such as neurite outgrowth preferential in the direction of fibril alignment [11].

Purification of collagen from animal and human tissues can be done successfully, but the lingering concern of immunogenicity and disease transmission remains. In collagen type I, most of the immunogenic character derives from the telopeptides, which can be efficiently removed by enzymatic treatment to eliminate most of the immunogenic character. It may not be possible to remove all of the immunogenicity of these non-human proteins, however; for example, in patients treated with a bone-promoting growth factor delivered in a bovine type I collagen matrix, a fraction of the subjects developed anti-bovine collagen antibodies [12]. One means to circumvent this problem is to use exclusively human proteins, although these also carry a certain risk of disease transmission. To avoid this risk, methods for the recombinant expression of collagen have been recently described [13], and recombinant human collagen types I and III are already commercially available.

Glycosaminoglycans

The structural proteins of the ECM, such as collagen (described above) and elastin (mentioned below), are augmented in their biomechanical and biochemical functions by the glycosaminoglycans (GAGs). In most cases, these are components of proteoglycans of the ECM, except in the case of hyaluronic acid which is not covalently attached to a protein component. These strongly anionic polymers absorb water, and this osmotic swelling provides compressive strength.

Hyaluronic acid can be isolated from animal tissue, such as the rooster comb, and can be isolated from microbial cultures. This material absorbs enormous amounts of water at equilibrium and eventually becomes fully soluble, owing to its high molecular weight (up to several million Da) it dissolves only very slowly. Several chemical derivatives have been presented aimed at rendering the polymer controllably more hydrophobic and thus less soluble (e.g. by grafting with hydrophobic esters [14]) or aimed at cross-linking the material into an elastic gel [15–17]. These materials have been used as barriers to prevent postoperative adhesion formation in internal healing [17] and to transplant cells for *in situ* tissue formation. Notably, the transplantation of both chondrocytes and mesenchymal stem cells within matrices constructed from hyaluronic acid derivatives has been reported in the repair of articular cartilage [18].

Purified chondroitin sulfate has also been purified from animal sources and utilized in matrices with collagen as a structural component in tissue regeneration [19]. Chondroitin sulfate–collagen composites have been used in repair of the skin [20] and peripheral nerves [21]. Moreover, it is apparent that the composite induces regenerative responses that can be more favorable than those induced by collagen alone. Whether this is due to interaction with growth factors, many of which have GAG-binding domains (see below), or the result of direct interaction with cells in the regeneration response is unclear.

Fibrin

Fibrin is a specialized ECM protein network, formed principally in spontaneous tissue repair. Although not an ECM in the usual sense, in that it is not produced by cells in the local environment, the material is nevertheless an important member of the body's repertoire of matrices and serves the role of a provisional matrix, being remodeled and replaced to form an ECM. Fibrin forms spontaneously by polymerization of fibrinogen in the presence of thrombin; the resulting network is further cross-linked by the transglutaminase activity of factor XIIIa [22]. Fibrin is a complex network, the fibril structure and cross-linked character depend upon the details of its formation [23,24]. In contrast to observations in fibrillar collagen matrices, cell migration in fibrin is almost exclusively dependent upon cell-associated proteolytic activity (e.g. from plasmin [25,26] and MMPs [27–29]). This distinction from behavior in fibrillar collagen probably results from the smaller mesh size of the fibrin matrices and the stronger fibril–fibril interactions, owing to the nature of network formation and covalent stabilization.

Human fibrin is clinically available from autologous sources and from cryoprecipitated pooled blood plasma. Fibrin has been utilized in skin repair, for example, in sutureless fixation of skin grafts [30] and keratinocyte transplantation in burn patients [31•], with considerable success. Fibrin has been explored extensively for use in growth factor release; for example, of members of the fibroblast growth factor (FGF) family in vascular graft engineering [32,33] and of the bone morphogenetic protein (BMP) superfamily [34]. To covalently endow fibrin matrices with the character of ECM molecules or other bioactive molecules, an enzymatic modification procedure has been described [35] (see below).

Mimicry of the fibrillar and viscoelastic character of the ECM

At one level, the ECM can be considered to be a network of tensile elements, consisting of fibrillar and amorphous

structural proteins, bi-continuous with a network of compressive elements, consisting of amorphous proteoglycans and GAGs. The collagens and elastin play the dominant role in providing tensile strength, and the hyaluronic acid plays a dominant role in most tissues in providing compressive strength. Several groups have attempted to mimic several aspects of the biomechanics of the ECM provided by these two elements.

Fibrillar matrices and nanoscale fibers

Fibrillar matrices formed from synthetic polymers, such as hydrolytically sensitive aliphatic polyesters, have been used extensively in tissue engineering, employing materials with fiber diameters on the order of tens of micrometers. These approaches are well reviewed elsewhere [36–38].

To extend fibrillar matrices towards the dimensions of the fibers found in the ECM, the process of electrospinning [39] has been applied to polyesters [40] and to actual biological materials, such as collagen [41,42] and fibrinogen [43]. Using such approaches, it is possible to obtain fiber diameters on the order of tens of nanometers. Although the incremental potential of such small-scale fibers has not yet been fully demonstrated in animal models, the modulation of fiber diameters might provide an approach to control the severity of the inflammatory response that is mounted by the body during materials resorption.

Elastic gel matrices

Elastic gel matrices have been explored in tissue engineering. A key limitation to the chemistry of these materials is how cells are placed in intimate, three-dimensional contact with the material. Because the mesh size of the networks is much smaller than a cell diameter, the cells must be introduced to the precursor of the gel matrix, rather than to the pre-formed gel matrix itself. This requires the development of approaches to gelation that are consistent with the toxicological limitations of living cells.

Alginate is one member of a broad class of polysaccharides that gels upon contact with divalent cations, especially calcium. Aqueous solutions of the sodium salt of alginate at 0.5–2% wt gel almost instantly upon contact with calcium ions in excess of 100 μM to form a stable, biocompatible elastic gel [44]. These materials have been widely employed to encapsulate and immunoisolate cells for transplantation [45,46] and to culture chondrocytes to form cartilage *in vitro* for articular reconstruction [47] and mechanical augmentation of soft tissues [48], for example. Because gelation occurs at physiological pH and temperature upon exposure to physiological or slightly superphysiological calcium ion concentrations, the cellular biocompatibility of the gelation process is excellent.

Water-soluble polymers that demonstrate lower or upper critical solution temperatures can also be employed to obtain gels, and cells can be evenly distributed within the precursors. Cooling or warming the materials induces the phase transition from the liquid to gel. These materials include the well-studied block copolymers of poly(ethylene glycol) and poly(propylene glycol) [49], which are liquids when cold but elastic gels at 37°C. The polymer within the gel phase of these materials exists in an equilibrium with the polymer in the soluble phase contacting the gel. Therefore, when such thermoreversible materials are placed within the open system of the body, polymer chains in the gel diffuse out of the gel into the fluids of the body, until eventually the gel completely dissolves.

To obtain elastic gels of synthetic water-soluble polymers that are stable *in vivo*, several approaches using chemical cross-linking have been studied. The toxicological limitations of the cells, which are mixed in the precursor components of the gel, require the pH to be near-neutral, the temperature to be less than around 39°C, organic solvents to be limited to very dilute levels, and toxic leaving groups to be avoided. Various schemes have been presented the use of visible light induced photopolymerization of acrylate-functionalized precursors [50,51], transglutaminase-induced cross-linking of peptide-functionalized precursors [52,53••], and the reaction of correspondingly co-reactive pairs of polymer precursors, such as by a Michael-type addition reaction [54,55,56••]. These materials have been employed, for example, in cell encapsulation [57] and in the generation of cartilage in culture [58,59]. Selected biopolymers have also been functionalized to permit chemical gelation in direct contact with cells, for example, by photocross-linking and photopolymerization of hyaluronic acid [59,60] and elastin-like peptides [61].

Mimicry of the molecular recognition of the ECM

In addition to mimicking the structural character of the ECM, many research groups have turned their attention to mimicking the biological recognition character of the ECM, yet without the use of biomolecules isolated from animals or humans. Biological recognition between the cells and their surrounding three-dimensional ECM milieu is bi-directional and dynamic: the ECM presents adhesion signals to cells; it provides bound growth factors, which cells liberate using locally activated enzymes at the cell surface; and the cells further induce local remodeling at the cell–ECM interface. Mimicking these molecular recognition events presents a considerable challenge.

Mimicking the cell adhesion character of the ECM

Since pioneering work to identify the domains on adhesion proteins of the ECM that mediate receptor–ligand adhesion, perhaps best represented by identification of

the RGD tripeptide of fibronectin and several other adhesion molecules [62], knowledge of the molecular interactions between cell-surface receptors and ECM adhesion molecules has blossomed [63]. This has led to several approaches to incorporate these domains into materials [64–66]. Many of these approaches are consistent with efforts to reconstitute the cell-adhesive character of the ECM in three-dimensional matrices for tissue engineering, and this short section presents only this subset of the much larger literature.

With regards to the surfaces of fibrillar materials, several approaches have been presented to incorporate adhesion peptides in a stable manner. Adsorption of peptides grafted to the termini of surface-active polymers possesses the strong advantage of processing ease, and this has been accomplished with polymers that adsorb both to hydrophobic surfaces [67] and to anionic surfaces, such as aliphatic polyesters after slight degradation [68]. With these approaches, it is possible to employ the adsorbing polymer to simultaneously passivate the surface against nonspecific interactions (e.g. by a poly(ethylene glycol) brush) and to induce specific adhesion to a targeted receptor family (e.g. with adhesion peptides grafted to the termini of the poly(ethylene glycol) chains). Approaches have also been presented in which the passivating and ligand-presenting polymer chains are embedded in the material surface [69–72], which can add an additional degree of stability.

Of particular interest is the quantity and morphology of the adhesion ligands presented. Whereas early work focused on the surface density of adhesion ligand required for stable cell adhesion [64], more recent work showed the presentation of ligands in a clustered configuration to be advantageous. Ten adhesion peptides presented in proximity to one another was found to be preferable to the homogeneous or random presentation of the same number of adhesion peptides [73••,74–76].

In matrices for tissue engineering, it is often desirable to incorporate cells directly in gel precursors, as described above. Approaches employing the photopolymerization of acrylated adhesion peptides [76] or Michael-type addition reactions with cysteine-containing adhesion peptides [54] have been presented. These concepts permit mixing of cells with a matrix precursor and an adhesion molecule component, rather in a mix-and-match manner, to easily create customized matrices for tissue in-growth [56••].

In addition to synthetic materials, it is also possible to incorporate adhesion ligands into biologically derived materials to alter their character. This has been explored with alginate derivatives, to add adhesion character to a material that presents little in its native state [77], and in fibrin matrices, to add adhesion character associated with ECM molecules that are exogenous to native fibrin [78,79]. Using such an approach, it was possible to incorporate several adhesion domains from the ECM protein laminin within fibrin and to enhance peripheral nerve regeneration in animal models [80•].

Given the structural complexity of some adhesion proteins, and the ability to use other biophysical forces to induce gelation, some groups have turned to the use of biosynthetic protein polymers as matrix materials. For example, endothelial cell-adhesive adhesion peptides have been engineered into elastin-like recombinant protein polymers to develop materials for vascular tissue engineering [81].

Mimicking the growth factor binding character of the ECM

One key function of the ECM in both morphogenesis and tissue repair is the presentation of growth factors. Growth factor interactions with anionic domains in the ECM modulate their partitioning from the ECM to the soluble phase, and thus control their local diffusion, signaling and dissipation. Growth factor–ECM interactions also contribute substantially to stabilizing the factor to provide prolonged activity. To mimic this behavior in materials for tissue engineering, growth factors have been covalently coupled [82,83] and electrostatically complexed [84•,85] to material matrices. For example, recombinant growth factor variants have been produced for incorporation into fibrin as a cell in-growth and tissue regeneration matrix. The variants have been expressed as fusion proteins with a domain that is a substrate for factor XIIIa, which cross-links the growth factor into fibrin during coagulation. A protease cleavage site is included between the growth factor domain and the fibrin-coupling site to enable localized release by proteases active at the surface of the cell. This approach has been used in materials for nerve regeneration [82,84•] and for induction of angiogenesis [86].

Mimicking the proteolytic sensitivity of the ECM

Cells migrate through the ECM, as well as through fibrin matrices in healing, by proteolytically clearing a pathway through the matrix to permit forward cell motion [27]. This is in contrast to the mechanisms of cell migration in collagen gels reconstituted *in vitro* under most conditions, and cell migration in macroporous fibrillar matrices and sponges. To mimic this behavior in a tissue engineering matrix, materials have been developed that are sensitive to the proteases expressed and activated at the surfaces of migrating cells, including hyaluronidase, plasmin and the MMPs.

Enzymatically mediated cell migration has been provided in materials formed from chemically and photochemically cross-linked hyaluronic acid [16,59,60]. Such materials may be particularly useful in the repair of cartilage defects. To design completely synthetic materials, two

useful approaches have been presented employing conjugates of poly(ethylene glycol) and peptides, the peptides being designed as substrates for plasmin or MMPs. In one approach, poly(ethylene glycol) is reacted on both termini with a peptide, and the peptide is further activated towards polymerization (e.g. by acrylation [83,87••]). Polymerization can be induced by visible light irradiation with non-toxic photosensitizers under conditions sufficiently gentle as to completely preserve cell viability. The network formed by polymerization consists of nodes of linear poly(acrylic acid) linked by chains of peptide-flanked poly(ethylene glycol). Thus, cell migration is permitted by proteolysis of the peptides, yielding completely soluble final products. Adhesion character can be inserted within this network by copolymerization with an acrylated adhesion peptide, such as an RGD sequence [76], and growth factors can also be incorporated [83,87••]. Reactively modified protein polymers have also been engineered to be photopolymerizable, permitting the creation of very complex, multifunctional materials [88].

A synthetically more versatile system has been recently described for forming synthetic networks that are proteolytically sensitive, bear adhesion peptides, and which also present entrapped or bound growth factors. A Michael-type addition reaction can be employed to cross-link multifunctional poly(ethylene glycol) with a di-cysteine peptide, the sequence of the peptide contains a protease substrate between the two cysteine residues [56••]. Adhesion peptides can be incorporated via reaction with mono-cysteine-containing adhesion peptides, and growth factors can be incorporated by entrapment within the network structure of the material or by reaction via free cysteine residues on a recombinantly engineered growth factor. This material presents the synthetic ease of simply mixing two peptides (the adhesion peptide and the cross-linking peptide) with a reactive poly(ethylene glycol). Incorporated cells retain their viability, spread in three dimensions and migrate, in all cases degrading the material only at their surfaces with a spatial fidelity in the order of 10–100 nm [56••]. These materials have been applied in bone repair, with an entrapped BMP to induce mesenchymal stem cell infiltration from the soft tissues and differentiation into chondrocytes and osteoblasts [89•,90].

Outlook and remaining challenges

Two factors have enabled biomaterials scientists to recapitulate many of the key characteristics of the ECM in practical material systems for tissue engineering: deeper understanding of the ECM and its biology and the development of methods to produce new materials. Using the approaches described above, it has become possible to mimic most of the features of the ECM, but not all. Spatial co-localization of different adhesion ligands or adhesion ligands and growth factor molecules remains difficult; given the fibrillar structure of the natural ECM, this feature may well be important in controlling cellular behavior. Likewise, *in situ* formation of materials that have both tensile fibrillar elements and compressive gel elements remains elusive: *in situ* gelation has recently been described using several approaches, but *in situ* fibril formation may only now be coming into reach (e.g. using the concepts of self-assembly [91••,92••]). Building such composite systems with components of different enzymatic sensitivity also remains complex.

In addition to the technical hurdles still to be overcome, there also exists a gap in our knowledge of the biological interactions between cells and the ECM in controlling cell differentiation and behavior in morphogenesis and repair. Exactly how growth factors and adhesion factors interact, both physically and within a biomechanical context, at present remains unclear. Indeed, model material systems might be required to experimentally elucidate these subtle interactions in culture and in animal models.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Knapp DM, Tower TT, Tranquillo RT, Barocas VH: **Estimation of cell traction and migration in an isometric cell traction assay.** *AIChE J* 1999, **45**:2628-2640.

2. •• Wolf K, Mazo I, Leung H, Engelke K, von Andrian UH, Deryugina EI, Strongin AY, Brocker EB, Friedl P: **Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis.** *J Cell Biol* 2003, **160**:267-277.

Working in collagen gels, the ability of cells to switch their paradigm for migration is demonstrated. Under some conditions, cells migrate employing proteolytic mechanisms in an elongated morphology, under other conditions they migrate in a non-proteolytic fashion in an amoeba-like morphology. The results have clear implications for materials design, specifically regarding nanofibrillar materials and protease-sensitive gels.

3. Grinnell F: **Fibroblast biology in three-dimensional collagen matrices.** *Trends Cell Biol* 2003, **13**:264-269.

4. Gabbiani G: **The myofibroblast in wound healing and fibrocontractive diseases.** *J Pathol* 2003, **200**:500-503.

5. Shreiber DI, Barocas VH, Tranquillo RT: **Temporal variations in cell migration and traction during fibroblast-mediated gel compaction.** *Biophys J* 2003, **84**:4102-4114.

6. Hinz B, Gabbiani G, Chaponnier C: **The NH2-terminal peptide of α-smooth muscle actin inhibits force generation by the myofibroblast *in vitro* and *in vivo*.** *J Cell Biol* 2002, **157**:657-663.

7. Girton TS, Oegema TR, Grassl ED, Isenberg BC, Tranquillo RT: **Mechanisms of stiffening and strengthening in media-equivalents fabricated using glycation.** *J Biomech Eng* 2000, **122**:216-223.

8. Uludag H, D'Augusta D, Golden J, Li J, Timony G, Riedel R, Wozney JM: **Implantation of recombinant human bone morphogenetic proteins with biomaterial carriers: a correlation between protein pharmacokinetics and osteoinduction in the rat ectopic model.** *J Biomed Mater Res* 2000, **50**:227-238.

9. Okamoto T, Yamamoto Y, Gotoh M, Liu D, Kihara M, Kameyama K, Hayashi E, Nakamura K, Yamauchi A, Huang CL *et al.*: **Cartilage regeneration using slow release of bone morphogenetic protein-2 from a gelatin sponge to treat experimental canine tracheomalacia: a preliminary report.** *ASAIO J* 2003, **49**:63-69.

10. Ceballos D, Navarro X, Dubey N, Wendelschafer-Crabb G,
• Kennedy WR, Tranquillo RT: **Magnetically aligned collagen gel filling a collagen nerve guide improves peripheral nerve regeneration.** *Exp Neurol* 1999, **158**:290-300.

The ability to induce alignment in fibrillar collagen matrices under magnetic fields is demonstrated. This alignment remains after the field is removed. Neurites are induced to migrate preferentially in the direction of fibril alignment, demonstrating local guidance by the material matrix.

11. Dubey N, Letourneau PC, Tranquillo RT: **Neuronal contact guidance in magnetically aligned fibrin gels: effect of variation in gel mechano-structural properties.** *Biomaterials* 2001, **22**:1065-1075.

12. Friedlaender GE, Perry CR, Cole JD, Cook SD, Cierny G, Muschler GF, Zych GA, Calhoun JH, LaForte AJ, Yin S: **Osteogenic protein-1 (bone morphogenetic protein-7) in the treatment of tibial nonunions.** *J Bone Joint Surg Am* 2001, **83A**:S151-S158.

13. Toman PD, Chisholm G, McMullin H, Gieren LM, Olsen DR, Kovach RJ, Leigh SD, Fong BE, Chang R, Daniels GA *et al.*: **Production of recombinant human type I procollagen trimers using a four-gene expression system in the yeast *Saccharomyces cerevisiae*.** *J Biol Chem* 2000, **275**:23303-23309.

14. Milella E, Brescia E, Massaro C, Ramires PA, Miglietta MR, Fiori V, Aversa P: **Physico-chemical properties and degradability of non-woven hyaluronan benzylic esters as tissue engineering scaffolds.** *Biomaterials* 2002, **23**:1053-1063.

15. Tomihata K, Ikada Y: **Crosslinking of hyaluronic acid with water-soluble carbodiimide.** *J Biomed Mater Res* 1997, **37**:243-251.

16. Bulpitt P, Aeschlimann D: **New strategy for chemical modification of hyaluronic acid: preparation of functionalized derivatives and their use in the formation of novel biocompatible hydrogels.** *J Biomed Mater Res* 1999, **47**:152-169.

17. Johns DB, Rodgers KE, Donahue WD, Kiorpes TC, diZerega GS: **Reduction of adhesion formation by postoperative administration of ionically cross-linked hyaluronic acid.** *Fertil Steril* 1997, **68**:37-42.

18. Solchaga LA, Goldberg VM, Caplan AI: **Cartilage regeneration using principles of tissue engineering.** *Clin Orthop* 2001, **391(Suppl)**:S161-S170.

19. Freyman TM, Yannas IV, Gibson LJ: **Cellular materials as porous scaffolds for tissue engineering.** *Prog Mater Sci* 2001, **46**:273-282.

20. Butler CE, Yannas IV, Compton CC, Correia CA, Orgill DP: **Comparison of cultured and uncultured keratinocytes seeded into a collagen-GAG matrix for skin replacements.** *Br J Plast Surg* 1999, **52**:127-132.

21. Chamberlain LJ, Yannas IV, Hsu HP, Strichartz GR, Spector M: **Near-terminus axonal structure and function following rat sciatic nerve regeneration through a collagen-GAG matrix in a ten-millimeter gap.** *J Neurosci Res* 2000, **60**:666-677.

22. Lorand L, Graham RM: **Transglutaminases: crosslinking enzymes with pleiotropic functions.** *Nat Rev Mol Cell Biol* 2003, **4**:140-156.

23. Ryan EA, Mockros LF, Stern AM, Lorand L: **Influence of a natural and a synthetic inhibitor of factor XIIIa on fibrin clot rheology.** *Biophys J* 1999, **77**:2827-2836.

24. Rocco M, Bernocco S, Turci M, Profumo A, Cuniberti C, Ferri F: **Early events in the polymerization of fibrin.** *Ann NY Acad Sci* 2001, **936**:167-184.

25. Herbert CB, Nagaswami C, Bittner GD, Hubbell JA, Weisel JW: **Effects of fibrin micromorphology on neurite growth from dorsal root ganglia cultured in three-dimensional fibrin gels.** *J Biomed Mater Res* 1998, **40**:551-559.

26. Herbert CB, Bittner GD, Hubbell JA: **Effects of fibrinolysis on neurite growth from dorsal root ganglia cultured in two- and three-dimensional fibrin gels.** *J Comp Neurol* 1996, **365**:380-391.

27. Werb Z: **ECM and cell surface proteolysis: regulating cellular ecology.** *Cell* 1997, **91**:439-442.

28. Vu TH, Werb Z: **Matrix metalloproteinases: effectors of development and normal physiology.** *Genes Dev* 2000, **14**:2123-2133.

29. Sternlicht MD, Werb Z: **How matrix metalloproteinases regulate cell behavior.** *Annu Rev Cell Dev Biol* 2001, **17**:463-516.

30. Currie LJ, Sharpe JR, Martin R: **The use of fibrin glue in skin grafts and tissue-engineered skin replacements: a review.** *Plast Reconstr Surg* 2001, **108**:1713-1726.

31. Horch RE, Bannasch H, Stark GB: **Transplantation of**
• **cultured autologous keratinocytes in fibrin sealant biomatrix to resurface chronic wounds.** *Transplant Proc* 2001, **33**:642-644.

Fibrin is demonstrated as an effective cell transplantation matrix in the repair of dermal burns with cells isolated from healthy skin. The clinical implications of this paper are substantial.

32. Tassiopoulos AK, Greisler HP: **Angiogenic mechanisms of endothelialization of cardiovascular implants: a review of recent investigative strategies.** *J Biomater Sci Polym Ed* 2000, **11**:1275-1284.

33. Xue L, Greisler HP: **Angiogenic effect of fibroblast growth factor-1 and vascular endothelial growth factor and their synergism in a novel *in vitro* quantitative fibrin-based 3-dimensional angiogenesis system.** *Surgery* 2002, **132**:259-267.

34. Mackenzie DJ, Sipe R, Buck D, Burgess W, Hollinger J: **Recombinant human acidic fibroblast growth factor and fibrin carrier regenerates bone.** *Plast Reconstr Surg* 2001, **107**:989-996.

35. Schense JC, Hubbell JA: **Cross-linking exogenous bifunctional peptides into fibrin gels with factor XIIIa.** *Bioconjug Chem* 1999, **10**:75-81.

36. Langer R: **Biomaterials in drug delivery and tissue engineering: one laboratory's experience.** *Acc Chem Res* 2000, **33**:94-101.

37. Vacanti JP, Langer R: **Tissue engineering: the design and fabrication of living replacement devices for surgical reconstruction and transplantation.** *Lancet* 1999, **354**:SI32-SI34.

38. Marler JJ, Upton J, Langer R, Vacanti JP: **Transplantation of cells in matrices for tissue regeneration.** *Adv Drug Deliv Rev* 1998, **33**:165-182.

39. Fridrikh SV, Yu JH, Brenner MP, Rutledge GC: **Controlling the fiber diameter during electrospinning.** *Phys Rev Lett* 2003, **90**:144502.

40. Yoshimoto H, Shin YM, Terai H, Vacanti JP: **A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering.** *Biomaterials* 2003, **24**:2077-2082.

41. Huang L, Nagapudi K, Apkarian RP, Chaikof EL: **Engineered collagen-PEO nanofibers and fabrics.** *J Biomater Sci Polym Ed* 2001, **12**:979-993.

42. Matthews JA, Wnek GE, Simpson DG, Bowlin GL: **Electrospinning of collagen nanofibers.** *Biomacromolecules* 2002, **3**:232-238.

43. Wnek GE, Carr ME, Simpson DG, Bowlin GL: **Electrospinning of nanofiber fibrinogen structures.** *Nano Lett* 2003, **3**:213-216.

44. Goosen MFA, King GA, McKnight CA, Marcotte N: **Animal-cell culture engineering using alginate polycation microcapsules of controlled membrane molecular-weight cutoffs.** *J Membr Sci* 1989, **41**:323-343.

45. Lim F, Sun AM: **Microencapsulated islets in diabetic rats.** *Science* 1981, **213**:1146-1146.

46. Soon-Shiong P: **Treatment of type I diabetes using encapsulated islets.** *Adv Drug Deliv Rev* 1999, **35**:259-270.

47. Wong M, Siegrist M, Wang XH, Hunziker E: **Development of mechanically stable alginate/chondrocyte constructs: effects of guluronic acid content and matrix synthesis.** *J Orthop Res* 2001, **19**:493-499.

48. Atala A, Kim W, Paige KT, Vacanti CA, Retik AB: **Endoscopic treatment of vesicoureteral reflux with a chondrocyte-alginate suspension.** *J Urol* 1994, **152**:641-643.

49. Cellesi F, Tirelli N, Hubbell JA: **Materials for cell encapsulation via a new tandem approach combining reverse thermal gelation and covalent crosslinking**. *Macromol Chem Phys* 2002, **203**:1466-1472.

50. Sawhney AS, Pathak CP, Hubbell JA: **Modification of islet of Langerhans surfaces with immunoprotective poly(ethylene glycol) coatings via interfacial photopolymerization**. *Biotechnol Bioeng* 1994, **44**:383-386.

51. Sawhney AS, Pathak CP, Hubbell JA: **Bioerodible hydrogels based on photopolymerized poly(ethylene glycol)-co-poly(α-hydroxy acid) diacrylate macromers**. *Macromolecules* 1993, **26**:581-587.

52. Sperinde JJ, Griffith LG: **Control and prediction of gelation kinetics in enzymatically cross-linked poly(ethylene glycol) hydrogels**. *Macromolecules* 2000, **33**:5476-5480.

53. •• Sanborn TJ, Messersmith PB, Barron AE: ***In situ* crosslinking of a biomimetic peptide-PEG hydrogel via thermally triggered activation of factor XIII**. *Biomaterials* 2002, **23**:2703-2710.
An extremely gentle process for *in situ* gelation of materials is described, utilizing synthetic polymers that are substrates for transglutaminases and a recombinant transglutaminase to induce cross-linking.

54. Elbert DL, Hubbell JA: **Conjugate addition reactions combined with free-radical cross-linking for the design of materials for tissue engineering**. *Biomacromolecules* 2001, **2**:430-441.

55. Elbert DL, Pratt AB, Lutolf MP, Halstenberg S, Hubbell JA: **Protein delivery from materials formed by self-selective conjugate addition reactions**. *J Control Release* 2001, **76**:11-25.

56. •• Lutolf MP, Raeber GP, Zisch AH, Tirelli N, Hubbell JA: **Cell-responsive synthetic hydrogels**. *Adv Mater* 2003, **15**:888-892.
A novel class of biointeractive gels is introduced. Gel precursors are mixed in the presence of cells, using a cross-linking chemistry that preserves cell viability. The resulting materials mimic key characteristics of the ECM: adhesion to cells, proteolytic sensitivity, and ability to entrap growth factors.

57. Cruise GM, Hegre OD, Lamberti FV, Hager SR, Hill R, Scharp DS, Hubbell JA: ***In vitro* and *in vivo* performance of porcine islets encapsulated in interfacially photopolymerized poly(ethylene glycol) diacrylate membranes**. *Cell Transplant* 1999, **8**:293-306.

58. Bryant SJ, Anseth KS: **Controlling the spatial distribution of ECM components in degradable PEG hydrogels for tissue engineering cartilage**. *J Biomed Mater Res* 2003, **64A**:70-79.

59. Park YD, Tirelli N, Hubbell JA: **Photopolymerized hyaluronic acid-based hydrogels and interpenetrating networks**. *Biomaterials* 2003, **24**:893-900.

60. Leach JB, Bivens KA, Patrick CW, Schmidt CE: **Photocrosslinked hyaluronic acid hydrogels: natural, biodegradable tissue engineering scaffolds**. *Biotechnol Bioeng* 2003, **82**:578-589.

61. Nagapudi K, Brinkman WT, Leisen JE, Huang L, McMillan RA, Apkarian RP, Conticello VP, Chaikof EL: **Photomediated solid-state cross-linking of an elastin-mimetic recombinant protein polymer**. *Macromolecules* 2002, **35**:1730-1737.

62. Ruoslahti E, Pierschbacher MD: **New perspectives in cell-adhesion — RGD and integrins**. *Science* 1987, **238**:491-497.

63. Sakiyama-Elbert SE, Hubbell JA: **Functional biomaterials: design of novel biomaterials**. *Annu Rev Mater Res* 2001, **31**:183-201.

64. Massia SP, Hubbell JA: **An RGD spacing of 440nm is sufficient for integrin α-V-β-3-mediated fibroblast spreading and 140nm for focal contact and stress fiber formation**. *J Cell Biol* 1991, **114**:1089-1100.

65. Hubbell JA: **Bioactive biomaterials**. *Curr Opin Biotechnol* 1999, **10**:123-129.

66. Hubbell JA: **Biomaterials in tissue engineering**. *Biotechnology* 1995, **13**:565-576.

67. Neff JA, Tresco PA, Caldwell KD: **Surface modification for controlled studies of cell–ligand interactions**. *Biomaterials* 1999, **20**:2377-2393.

68. VandeVondele S, Voros J, Hubbell JA: **RGD-Grafted poly-l-lysine-graft-(polyethylene glycol) copolymers block non-specific protein adsorption while promoting cell adhesion**. *Biotechnol Bioeng* 2003, **82**:784-790.

69. Drumheller PD, Hubbell JA: **Densely cross-linked polymer networks of poly(ethylene glycol) in trimethylolpropane triacrylate for cell-adhesion-resistant surfaces**. *J Biomed Mater Res* 1995, **29**:207-215.

70. Banerjee P, Irvine DJ, Mayes AM, Griffith LG: **Polymer latexes for cell-resistant and cell-interactive surfaces**. *J Biomed Mater Res* 2000, **50**:331-339.

71. Desai NP, Hubbell JA: **Solution technique to incorporate polyethylene oxide and other water-soluble polymers into surfaces of polymeric biomaterials**. *Biomaterials* 1991, **12**:144-153.

72. Bearinger JP, Castner DG, Healy KE: **Biomolecular modification of p(AAm-co-EG/AA) IPNs supports osteoblast adhesion and phenotypic expression**. *J Biomater Sci Polym Ed* 1998, **9**:629-652.

73. •• Maheshwari G, Brown G, Lauffenburger DA, Wells A, Griffith LG: **Cell adhesion and motility depend on nanoscale RGD clustering**. *J Cell Sci* 2000, **113**:1677-1686.
Clustering of adhesion ligands is shown to induce qualitatively and quantitatively different cell adhesion than the same adhesion ligand density distributed randomly on a surface. Implications for materials design are substantial.

74. Irvine DJ, Ruzette AVG, Mayes AM, Griffith LG: **Nanoscale clustering of RGD peptides at surfaces using comb polymers. 2. Surface segregation of comb polymers in polylactide**. *Biomacromolecules* 2001, **2**:545-556.

75. Irvine DJ, Mayes AM, Griffith LG: **Nanoscale clustering of RGD peptides at surfaces using comb polymers. 1. Synthesis and characterization of comb thin films**. *Biomacromolecules* 2001, **2**:85-94.

76. Hern DL, Hubbell JA: **Incorporation of adhesion peptides into nonadhesive hydrogels useful for tissue resurfacing**. *J Biomed Mater Res* 1998, **39**:266-276.

77. Kong HJ, Smith MK, Mooney DJ: **Designing alginate hydrogels to maintain viability of immobilized cells**. *Biomaterials* 2003, **24**:4023-4029.

78. Schense J, Hubbell J: **Cross-linking exogenous bifunctional peptides into fibrin gels with factor XIIIa**. *Bioconjug Chem* 1999, **10**:75-81.

79. Schense JC, Hubbell JA: **Three-dimensional migration of neurites is mediated by adhesion site density and affinity**. *J Biol Chem* 2000, **275**:6813-6818.

80. • Schense JC, Bloch J, Aebischer P, Hubbell JA: **Enzymatic incorporation of bioactive peptides into fibrin matrices enhances neurite extension**. *Nat Biotechnol* 2000, **18**:415-419.
The character of an ECM molecule, laminin, is conferred upon a natural healing matrix, fibrin. Improved peripheral nerve regeneration is observed *in vivo*.

81. Panitch A, Yamaoka T, Fournier MJ, Mason TL, Tirrell DA: **Design and biosynthesis of elastin-like artificial extracellular matrix proteins containing periodically spaced fibronectin CS5 domains**. *Macromolecules* 1999, **32**:1701-1703.

82. Sakiyama-Elbert SE, Panitch A, Hubbell JA: **Development of growth factor fusion proteins for cell-triggered drug delivery**. *FASEB J* 2001, **15**:1300-1302.

83. DeLong SA, Mann BK, West JL: **Scaffolds modified with tethered growth factors to influence smooth muscle cell behavior**. *FASEB J* 2002, **16**:A36-A36.

84. • Sakiyama-Elbert SE, Hubbell JA: **Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix**. *J Control Release* 2000, **69**:149-158.
Materials are formed that function *in vivo* essentially as affinity adsorption matrices, binding growth factors through bound heparin and releasing that growth factor as cells invade the matrix, locally degrading it.

85. Sakiyama-Elbert SE, Hubbell JA: **Development of fibrin derivatives for controlled release of heparin-binding growth factors**. *J Control Release* 2000, **65**:389-402.

86. Zisch AH, Schenk U, Schense JC, Sakiyama-Elbert SE, Hubbell JA: **Covalently conjugated VEGF-fibrin matrices for endothelialization**. *J Control Release* 2001, **72**:101-113.

87. •• Gobin AS, West JL: **Cell migration through defined, synthetic extracellular matrix analogues**. *FASEB J* 2002, **16**:751-753.
Photopolymerization of a functional PEG–peptide copolymer is used to form matrices into which cells can invade under the chemotactic influence of a growth factor.

88. Halstenberg S, Panitch A, Rizzi S, Hall H, Hubbell JA: **Biologically engineered protein-graft-poly(ethylene glycol) hydrogels: a cell adhesive and plasmin-degradable biosynthetic material for tissue repair**. *Biomacromolecules* 2002, **3**:710-723.

89. • Lutolf MR, Weber FE, Schmoekel HG, Schense JC, Kohler T, Muller R, Hubbell JA: **Repair of bone defects using synthetic mimetics of collagenous extracellular matrices**. *Nat Biotechnol* 2003, **21**:513-518.
The biointeractive gels mentioned elsewhere are capable of inducing bone healing in animal models as effectively as natural, biologically derived materials.

90. Lutolf MP, Lauer-Fields JL, Schmoekel HG, Metters AT, Weber FE, Fields GB, Hubbell JA: **Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: engineering cell-invasion characteristics**. *Proc Natl Acad Sci USA* 2003, **100**:5413-5418.

91. •• Kisiday J, Jin M, Kurz B, Hung H, Semino C, Zhang S, Grodzinsky AJ: **Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: implications for cartilage tissue repair**. *Proc Natl Acad Sci USA* 2002, **99**:9996-10001.
Using self-assembling peptides, it is possible to assemble a number of different material morphologies, ranging from amorphous to nanofibrillar. The assembly process can be sufficiently gentle as to induce the transformation *in situ*, in the presence of cells. Here, application in chontrocyte transplantation and cartilage repair is highlighted.

92. •• Zhang SG, Marini DM, Hwang W, Santoso S: **Design of nanostructured biological materials through self-assembly of peptides and proteins**. *Curr Opin Chem Biol* 2002, **6**:865-871.
The ability to form a number of gel nanomorphologies through self-assembly of peptide precursors is demonstrated. The gentleness of the transformation conditions is conducive to *in situ* conversion from liquids to gels.